METHODS IN MEDICAL RESEARCH

Volume 4 · M. B. VISSCHER, *Editor-in-Chief*

Governing Board
IRVINE H. PAGE
A. C. IVY
COLIN M. MACLEOD
CARL F. SCHMIDT
EUGENE A. STEAD
DAVID L. THOMSON

CFTRI-MYSORE
3538
Methods in medic

[illegible]

.00
1573/-

Accn. 3538

Tiv

11/58

METHODS IN
MEDICAL RESEARCH

Volume 4

METHODS IN MEDICAL RESEARCH

VOLUME 1

VAN R. POTTER, *Editor-in-Chief*

ASSAY OF ANTIBIOTICS, *Henry Welch, Editor*
CIRCULATION—BLOOD FLOW MEASUREMENT, *Harold D. Green, Editor*
SELECTED METHODS IN GASTROENTEROLOGIC RESEARCH, *A. C. Ivy, Editor*
CELLULAR RESPIRATION, *Van R. Potter, Editor*

VOLUME 2

JULIUS H. COMROE, JR., *Editor-in-Chief*

METHODS OF STUDY OF BACTERIAL VIRUSES, *Mark H. Adams, Editor*
PULMONARY FUNCTION TESTS, *Julius H. Comroe, Jr., Editor*
ASSAY OF HORMONAL SECRETIONS, *Eleanor H. Venning, Editor*

VOLUME 3

RALPH W. GERARD, *Editor-in-Chief*

GENETICS OF MICRO-ORGANISMS, *S. E. Luria, Editor*
ASSAY OF NEUROHUMORS, *J. H. Gaddum, Editor*
SELECTED PSYCHOMOTOR MEASUREMENT METHODS, *Walter R. Miles, Editor*
METHODS FOR STUDY OF PEPTIDE STRUCTURE, *Choh Hao Li, Editor*

METHODS IN
Medical Research

GOVERNING BOARD

Irvine H. Page, *Chairman;* A. C. Ivy; Colin M. MacLeod;
Carl F. Schmidt; Eugene A. Stead; David L. Thomson

Volume 4

MAURICE B. VISSCHER, *Editor-in-Chief*

HISTOCHEMICAL STAINING METHODS, *George Gomori, Editor*

FLUID AND ELECTROLYTE DISTRIBUTION, *Louis B. Flexner, Editor*

STUDIES ON GASTROINTESTINAL PRESSURES, INNERVATION AND SECRETIONS,
J. P. Quigley, Editor

TISSUE CULTURE METHODS, *C. M. Pomerat, Editor*

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, * MYSORE. *
15-12.1956
LIBRARY

THE YEAR BOOK PUBLISHERS, INC.
200 EAST ILLINOIS STREET, CHICAGO

COPYRIGHT 1951 BY THE YEAR BOOK PUBLISHERS, INC.

3538 ✓

L:f"n1,N

N51

CFTRI-MYSORE
3538
Methods in medic..

PRINTED IN U.S.A.

PREFACE

We are glad to feel that the hopes with which this series of volumes was launched have not proved fantastic. Many appreciative comments have reached us, and much of what criticism we have heard has been constructive and most welcome, and we have not been disappointed in our expectation that the appearance of each successive volume would stimulate some revived demand for its predecessors.

Dr. Maurice Visscher, who has so kindly and so effectively acted as Editor-in-Chief for the present volume, has in his own preface re-stated the purpose and the principles of the series. We have seen no need to depart from those originally laid down, though we have gained some experience in distinguishing the difficult from the impossible. In the summer of 1950 it seemed reasonable to believe that the series would continue for years and would attain a regular annual rhythm of production; in that belief, plans were laid, at least in outline, for some years ahead, so that editors and contributors might have more time in which to develop their ideas. If the future looks more uncertain today, that is because of the international situation rather than because of any vacillation on our part or any epidemic of unwillingness among those invited to contribute. Not only the members of the Governing Board but all users of these volumes are indebted to their fellow-scientists who have generously given time and thought to make their technical knowledge accessible to all.

Irvine H. Page
A. C. Ivy
Colin M. MacLeod
Carl F. Schmidt
Eugene A. Stead
David L. Thomson

EDITOR'S PREFACE

The rate of progress of science is limited by many controlling factors, some of which are not readily subject to alteration. One of the crucial determinants of the rate is the accessibility of reliable information about working methods for use in research. It is in order to improve the availability of such information for the field of medical investigation that this series of volumes is being published.

There are 2 important reasons why it is justifiable to ask busy investigators to assist in preparing these books. The first is that, in general, methodology is usually relegated to a place of smaller type and sharply abbreviated importance in journal publication of research. Numerous essential details are customarily omitted, either because they are considered to be common knowledge or simply for lack of space. Such details are frequently common knowledge to the experienced worker in the field, but to the novice they are not. And it is to the novice of today that one must obviously look for the substantial production of tomorrow.

The second basis of the need for special collections of material on methodology is the rise of specialization with the growth of science. There is obviously much virtue in concentration of effort provided the scientist is not prevented from full exploration of a field because of unfamiliarity with the variety of methods of study which would be useful and essential to such full study. There is no doubt that in many cases a lack of knowledge about methods is at least as serious a handicap to research as the unavailability of apparatus. With the tremendous increase which the last 50 years have seen in the variety and number of methods of scientific study, it has become imperative that the methodology of science be given special treatment. The substantive discoveries of medical science are published in many thousands of periodicals. To be sure, the existing methods of primary and secondary publication of the observations of science are not satisfactory, but they are much more nearly so than are our present means for making the facts of methodology available. In this era of specialization the need for detailed descriptions of working procedures by persons who have had large practical experience with them is obvious. Without this development specialization would lead to sterility.

Related to the rise of specialization is the prodigious increase in the number of methods employed in scientific study. It has become impractical and in fact impossible to train workers in a field in more than a small fraction of the methods pertinent to the field during a reasonable period of undergraduate and graduate study.

It is the hope of the authors, editors and Governing Board that this volume and the series of which it is a part may contribute to the progress of medical science by meeting some of the needs outlined. The major credit for the undertaking must be given to the contributors for their generous spirit in making their own practical experiences available to others through this medium.

—Maurice B. Visscher.

CONTRIBUTORS AND REVIEWERS

BIESELE, JOHN J., Ph.D.

Head, Cell Growth Section, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York.

BRODIE, BERNARD B., Ph.D.

Section of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Md.

BRODY, DANIEL A., M.D.

Division of Physiology, University of Tennessee, Memphis.

BRUES, AUSTIN M., M.D.

Director, Division of Biological and Medical Research, Argonne National Laboratory; Associate Professor of Medicine, Department of Medicine and Institute of Radiobiology and Biophysics, University of Chicago, Chicago.

BUCHSBAUM, RALPH, Ph.D.

Professor of Zoology, Department of Biological Sciences, University of Pittsburgh, Pittsburgh.

CHINARD, FRANCIS P., M.D.

Instructor in Medicine, Departments of Medicine and Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore.

CODE, C. F., M.D.

Professor of Physiology, Mayo Clinic Foundation and Mayo Clinic, Rochester, Minn.

DAVENPORT, HORACE W., Ph.D.

Professor and Head, Department of Physiology, University of Utah Department of Medicine, Salt Lake City.

EARLE, WILTON R., Ph.D.

Head, Tissue Culture Unit, Biology Section, National Cancer Institute, National Institutes of Health, Bethesda, Md.

EDER, HOWARD A., M.D.

Assistant Professor of Medicine, Cornell University Medical College, New York.

ELMAN, ROBERT, M.D.

Professor of Surgery, Washington University School of Medicine, St. Louis.

EVANS, VIRGINIA J., Sc.D.

Cytologist, Tissue Culture Unit, Biology Section, National Cancer Institute, National Institutes of Health, Bethesda, Md.

FELL, HONOR B., D.Sc., Ph.D.

Foulerton Research Fellow, Royal Society; Director, Strangeways Research Laboratory, Cambridge, England.

FENN, W. O., Ph.D.

Professor of Physiology, Schools of Dentistry and Medicine, University of Rochester, Rochester, N. Y.

FLEXNER, LOUIS B., M.D.

Department of Embryology, Carnegie Institution of Washington, Baltimore.

FRIEDMAN, M. H. F., Ph.D.

Professor of Physiology, Jefferson Medical College, Philadelphia.

GAILLARD, PETER J., M.D.

Professor of Experimental Histology, State University of Leyden, Leyden, The Netherlands.

GOMORI, GEORGE, M.D.

Professor of Medicine, University of Chicago, Chicago.

GREGERSEN, M. I., Ph.D.

Professor of Physiology, College of Physicians and Surgeons, Columbia University, New York.

GROSSMAN, M. I., Ph.D., M.D.

Professor of Physiology, Department of Clinical Science, University of Illinois Medical School, Chicago.

HALD, PAULINE M., B.A.

Assistant Professor of Medicine, Yale University School of Medicine, New Haven, Conn.

HASTINGS, A. B., Ph.D.

Professor of Biochemistry, Harvard Medical School, Boston.

HETHERINGTON, DUNCAN C., Ph.D., M.D.

Professor of Anatomy, Duke University School of Medicine, Durham, N. C.

HOLLANDER, FRANKLIN, Ph.D.

Head, Gastroenterology Research Laboratory, Mount Sinai Hospital, New York.

McLEAN, FRANKLIN C., M.D.

Professor of Pathological Physiology, University of Chicago, Chicago.

MANERY, J. F., Ph.D.

Assistant Professor of Biochemistry, University of Toronto, Toronto, Canada.

MARTINOVITCH, PETAR N., Ph.D.

Director, Laboratory for Experimental Biology, Serbian Academy of Science, Novi Sad, Yugoslavia.

MEYER, HERTHA

Head, Tissue Culture Laboratory, Institute of Biophysics, University of Brazil, Rio de Janeiro.

MURRAY, MARGARET R., Ph.D.

Assistant Professor of Surgery, College of Physicians and Surgeons, Columbia University, New York.

PACE, NELLO, Ph.D.

Assistant Professor of Physiology, University of California, Berkeley.

PETERS, JOHN P., M.D.

Professor of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

POMERAT, CHARLES M., Ph.D.

Professor of Cytology and Director, Tissue Culture Laboratory, University of Texas, Medical Branch, Galveston.

PORTER, KEITH R., Ph.D.

Associate Member, Rockefeller Institute for Medical Research, New York.

QUIGLEY, J. P., M.D.

Chief, Division of Physiology, University of Tennessee, Memphis.

RICHARDS, OSCAR W., Ph.D.

Research Supervisor for Biology, Research Laboratory, American Optical Company, Stamford, Conn.

SANFORD, KATHERINE K., Ph.D.

Cytologist, Tissue Culture Unit, Biology Section, National Cancer Institute, National Institutes of Health, Bethesda, Md.

STROUD, AGNES N., B.S.

Junior Biologist, Division of Biological and Medical Research, Argonne National Laboratory, Chicago.

THOMAS, J. E., M.D.

Professor of Physiology, Jefferson Medical College, Philadelphia.

VISSCHER, MAURICE B., M.D., Ph.D.

Professor and Head, Department of Physiology, University of Minnesota Medical School, Minneapolis.

WAYMOUTH, CHARITY, Ph.D.

Member of Scientific Staff, Chester Beatty Research Institute, Royal Cancer Hospital, London, England.

WHITE, PHILIP R., Ph.D.

Head, Department of General Physiology, Institute for Cancer Research, Philadelphia.

TABLE OF CONTENTS

SECTION I. Histochemical Staining Methods

ASSOCIATE EDITOR, *George Gomori*

Introduction 1
Inorganic Substances 3
  Calcium 3
  Iron 4
  Copper 5
  Zinc 5
  Lead 6
  Bismuth 6
  Mercury, Silver, Gold 6
Organic Substances 7
  Ascorbic Acid 7
  Arginine 7
  Uric Acid and Urates 7
  Adrenaline 8
  Phenolic Substance of Enterochromaffin Cells 8
  Polysaccharides 9
  Nucleic Acids 12
  Lipids 12
  Oxidative Enzymes 14
  Hydrolytic Enzymes 16
    Alkaline Phosphatase 17
    Acid Phosphatase 19
    Esterase and Lipase 20
    Cholinesterase 22
    Phosphamidase 23
Buffers for Use in Histochemistry 25

SECTION II. Fluid and Electrolyte Distribution

ASSOCIATE EDITOR, *Louis B. Flexner*

Introduction 29

Measurement of Total Body Water, by Bernard B. Brodie (comment by Nello Pace) 31
Estimation of Plasma Volume by Dye Dilution Method, by Francis P. Chinard 38
Determination of Thiocyanate Space, by Howard A. Eder 48
Radioisotope Method for Determining Volume of Extracellular Fluid in the Animal Body, by J. F. Manery 53
Biologic Method for Estimation of Calcium Ion Concentration, by Franklin C. McLean 68
Determinations with Flame Photometer, by Pauline M. Hald . . . 79

SECTION III. Studies on Gastrointestinal Pressures, Innervation and Secretions

ASSOCIATE EDITOR, *J. P. Quigley*

Introduction 106
Registration of Digestive Tract Intralumen Pressures, by Daniel A. Brody and J. P. Quigley 109
Preparation of Esophageal Fistulas and Gastric Pouches, by M. H. F. Friedman 123
Methods for Study of External Secretory Function of the Pancreas, by J. E. Thomas (comments by Robert Elman and L. R. Dragstedt) 149
Insulin-Acidity Test for Determining Integrity of Vagal Influence on the Stomach, by Franklin Hollander 166
Methods of Assaying Gastric Secretory Inhibitors, by M. I. Grossman (comment by C. F. Code) 173
Methods for in Vitro Study of Gastric Secretion, by Horace W. Davenport (comment by L. E. Edwards) 185

SECTION IV. Tissue Culture Methods

ASSOCIATE EDITOR, *C. M. Pomerat*

Introduction 198
Tissue Culture Media 201
  I. Approaches to Development of Synthetic Media for the Animal Cell 201
    Introduction, by Philip R. White 201
    A. Analytic and Supplementation Methods, by Charity Waymouth 201
    B. True Synthetic Media, by Philip R. White 206
  II. Commercial Media, by Duncan C. Hetherington 209

Tissue Culture Procedures in Medical Installations 211
A. Sources and Handling of Material, by Margaret R. Murray . 211
B. Double Cover Slip Culture Technique, by Margaret R. Murray 212
General Methods for Quantitative Studies on Cells in Tissue Culture 217
Method for Growth of Single Isolated Tissue Cells and Development of Pure Cell Strains, by Wilton R. Earle 217
Method for Growth of Cells on Perforated Cellophane, by Wilton R. Earle 218
Measurement of Proliferation of Tissue Cultures by Enumeration of Cell Nuclei, by Katherine K. Sanford 219
Preparation and Handling of Replicate Tissue Cultures for Quantitative Studies, by Virginia J. Evans 221
Measurement of Growth by Nucleic Acid Determinations, by Charity Waymouth 225
Methods for Study of Organized Growth in Vitro 233
Introduction, by Honor B. Fell 233
Techniques of Bone Cultivation, by Honor B. Fell 234
Culture of Infantile Endocrine Glands of Rats by Watch Glass Technique in a Moist Chamber, by Petar N. Martinovitch 237
Technique of Grafting Tissues and Organs, Including Those Cultivated in Vitro, into Anterior Eye Chamber of Rats and Mice, by Petar N. Martinovitch 240
Organ Culture Technique Using Embryologic Watch Glasses, by Peter J. Gaillard 241
Action of Physical Agents on Living Cells 247
Radioactive Tracers in Tissue Culture, by Austin M. Brues and Agnes N. Stroud 247
Methods of Studying Effects of Radiations on Tissue Cultures, by Ralph Buchsbaum 255
Action of Chemical Agents on Living Cells, by C. M. Pomerat . . 260
I. General Considerations in Selecting Testing Methods . . . 261
A. Use of Primary Explants vs. Cell Strains, by C. M. Pomerat 261
B. Methods of Culturing, by C. M. Pomerat 261
C. End-Points in Evaluating Effect of Chemicals on Cells, by C. M. Pomerat (comment by John J. Biesele) . 262
II. Use of Tissue Cultures in Drug-Testing Operations 266
A. Primary Outgrowth on Cover Slip Preparations, by C. M. Pomerat 266
B. Medawar's Hanging Drop Assay Test, by C. M. Pomerat 271
C. Flask Method (Parker), by C. M. Pomerat 271
D. Titration of Toxic or Lethal End-Points (Hanks), by C. M. Pomerat 271

E. Tissue Culture Screening of Agents in Experimental Cancer Chemotherapy, by John J. Biesele . . . 272
III. Cytochemical Studies 275
Perfusion Chamber, by C. M. Pomerat 275
Cell Parasite Relations in Tissue Culture 281
Cultivation of Intracellular Protozoa, by Hertha Meyer 281
Apparatus for Examining Cells Cultivated in Vitro 284
Phase Microscopy for Tissue Culture, by Oscar W. Richards . . 284
Electron Microscopy of Cultured Tissue Cells, by Keith R. Porter 288
Indexes 292

SECTION 1

Histochemical Staining Methods

ASSOCIATE EDITOR—*George Gomori*

INTRODUCTION

WITH A FEW exceptions, histochemistry utilizes the usual methods of analytical chemistry for the identification of chemical compounds in the tissues. Since exact microscopic localization is one of its main purposes, the preservation of the structural integrity of the tissue to be examined is of paramount importance. This fact rules out the use of many good methods which utilize such reagents as would destroy tissue structure (conc. H_2SO_4, strong alkali).

For a satisfactory localization, 2 conditions must be fulfilled. First, all displacement of the chemical substance, the localization of which is to be investigated, must be prevented. (1) In the case of insoluble or nondiffusible substances there is no danger of significant displacement; however, in the case of highly diffusible substances gross shifts can be prevented only by the use of the freezing-drying technique. (2) The product of the analytical reaction which must be specific must also be insoluble and, preferably, highly colored or at least convertible to a colored substance.

In addition to the regular chemical reactions whose nature is known, a number of staining techniques, the mechanisms of which are poorly understood, are also included among the methods of histochemistry because they will identify chemically more or less well defined substances such as glycogen and mucin. On the other hand, purely morphologic staining methods, useful as they may be in the differentiation of certain structures, are outside the scope of histochemistry because they do not give information of chemical nature.

The histochemical methods described here include only such techniques as (1) can be carried out with the routine apparatus available in an average well equipped laboratory of histology, and (2) are reasonably specific, sensitive and dependable. Methods requiring special apparatus such as freezing-drying equipment, microincinerator and spark spectrograph are not included. The methods described are not necessarily the original ones referred to; many of them are personal modifications. For convenience of reference, the values for buffers used in the various procedures are listed at the end of this section.

—George Gomori.

INORGANIC SUBSTANCES

Reactions have been described for a number of metallic elements, for arsenic, and also for chloride, phosphate and carbonate ions. The freezing-drying technique is required for the localization of K^+, Cl^-, and for dissolved $CO_3^=$ and $PO_4^=$ (or $HPO_4^=$) ions. The technique suggested for As lacks specificity. Methods described for the demonstration of Mg are not sufficiently sensitive for use on tissues of higher animal species. This leaves only Ca, Fe, Cu, Zn, Pb, Bi, Hg and Au to be discussed.

Calcium

The histochemical method can be applied only to insoluble deposits consisting, for all practical purposes, of a mixture of Ca phosphate and carbonate. To avoid loss of Ca, the tissue must be fixed either in neutralized Formalin or in 80–95% alcohol. The only absolutely specific test for Ca follows.

PROCEDURE

Put a few drops of 2–5% sulfuric acid in 50% alcohol on the slide and place a cover slip on it. If Ca is present, typical crystals of gypsum will form in a few minutes. The crystals are too coarse for exact localization, and counterstaining is not possible.

Since insoluble deposits of Ca in the tissues consist almost exclusively of phosphate and carbonate and, conversely, practically all insoluble phosphates and carbonates are those of Ca, a test for phosphate and carbonate is sufficiently specific for Ca. The method of Kóssa (21) and its modifications can be used for demonstration of the latter anions.

PROCEDURE

Wash the slide thoroughly in distilled water. Place it in a 0.2–0.5% (concentration not important) filtered solution of $AgNO_3$ for about 10 min. Calcium phosphate and carbonate are converted into the corresponding Ag salts which subsequently can be reduced to black metallic Ag either by performing the test in bright sunlight (or under an ultraviolet light source) or by the use of photographic developers. In the former case, the centers of positive reaction will often be surrounded by a disturbing yellowish brown halo. The photographic developers give a sharper, cleaner picture. It is important to wash the slide very thoroughly in repeated changes of distilled water before applying the developer; otherwise one may obtain a fine dustlike black precipitate all over the slide. From the last change of distilled water the slide is transferred into a freshly prepared dilute solution (about 0.2–0.5%; concentration not important) of any photographic developer (Elon, hydroquinone, Acrol, etc.) for 2–3 min. Sulfite should not be used because it may cause blurring of the picture. Rinse in distilled water. Whether daylight or chemical reduction is chosen, the slide must be fixed in a 1–2% solution of Na thiosulfate for 1 min (not longer!) to remove unreacted silver which may cause delayed darkening of the slide. Sites of calcification are jet black. Counterstain as desired; dehydrate and mount.

The only source of error may arise from the presence of uric acid or its salts which may also blacken with this method, even before application of the reducer. In case of doubt, they can be removed by treating the slide (before the $AgNO_3$ step) with a saturated solution of lithium carbonate, followed by thorough rinsing in distilled water. Calcifications, on the other hand, are easily removed by keeping the slide for about 2 min in a 0.25% solution of HNO_3. Melanin also will blacken on more prolonged exposure but is visible even before Ag treatment.

Iron

Iron occurs in 2 forms in the tissues. One type of compound, exemplified by hemosiderin, behaves chemically much like any poorly soluble inorganic compound of iron, readily demonstrable by the reactions of analytic chemistry. In the other type, of which hemoglobin is an example, iron can be demonstrated only after the destruction of the organic part of the molecule ("masked iron"). Apparently all iron found in the tissues is present in the trivalent form.

Hemosiderin-iron can best be demonstrated by the *Prussian blue method* (2).

PROCEDURE

Fix in alcohol, Formalin-alcohol (1:4) or (briefly) in Bouin's fluid. Long exposure of the tissue to acid-containing fixatives may result in false localization due to secondary adsorption of dissolved iron.

Run the sections to distilled water. Immerse them in a freshly prepared and filtered 5–10% solution of potassium ferrocyanide.

After a few minutes add about $^1/_{10}$–$^1/_{20}$ vol of concentrated hydrochloric acid (best analytic grade) and stir the mixture. Hemosiderin will start to turn greenish almost immediately, and in 10–20 min the reaction is complete. Wash the slide under the tap; counterstain with a red nuclear stain (carmine, safranine, etc.); dehydrate and mount. Iron shows up blue. Balsam of Canada will cause gradual fading of the stain. Use synthetic media (clarite, etc.) for permanent results.

Some forms of "masked" iron can be demasked by applying 30% (!) H_2O_2 to the section for about 10 min. Hemoglobin will become positive by the Prussian blue reaction after this treatment but, owing to its low iron content, will show up in a rather pale green-blue shade.

Copper

The forms in which copper occurs in higher animal organisms are poorly known. The following technique (31) will demonstrate Cu (possibly a cupric analogue of hemosiderin) in the organs of some molluscs and insects and occasionally in cirrhotic livers of human beings. On the other hand, livers proved chemically to be rich in Cu may be entirely negative histochemically.

PROCEDURE

Fix in alcohol or Formalin. Incubate the sections for about 24 hr at 37 C in a mixture of 50 ml of 5% aqueous sodium acetate and 2–5 ml of a 0.1% solution of rubeanic acid (dithiooxamide; Eastman #4394) in absolute alcohol. Wash under the tap. Counterstain with a red nuclear stain. Dehydrate and mount. Copper will be stained green-black.

Zinc

The following technique (32) allegedly demonstrates zinc in the tissues (especially pancreatic islets) of higher animals. The results obtained in our laboratory were erratic.

PROCEDURE

Fix in Formalin. Incubate the sections in a mixture of 50 ml of a NH_4OH–NH_4Cl buffer of pH 8–9 and 2–3 ml of a 2–3 day old saturated solution of dithizone (diphenylthiocarbazone; Eastman #3092; Matheson #4205) for 4–5 hr. Wash under the tap. Counterstain with hematoxylin. Mount in glycerin jelly. Zinc stains in a purplish red shade.

Lead

Lead can be demonstrated by fixation in any dichromate-containing medium (9). Insoluble $PbCrO_4$ will form which, although yellow, is quite dense in transmitted light. If desired, it can be transformed into brown-black PbS by treatment with H_2S or ammonium sulfide.

Bismuth

Bismuth is demonstrated histochemically in the form of Bi-brucine-iodide (37). Since Bi occurs in the tissues in the form of a blackish, nonreactive precipitate (Bi_2S_3?), it must be transformed first into a reactive form. This is accomplished by oxidation with H_2O_2.

PROCEDURE

Fix in Formalin or alcohol and prepare frozen or paraffin sections.

Prepare the reagent by dissolving 0.25 g of brucine sulfate, 2–3 drops of concentrated sulfuric acid and 2 g of KI in 100 ml of water.

Bleach the section for 1 min with 30% (!) H_2O_2. Rinse. Transfer into the reagent for 1 hr. Transfer into reagent diluted 1:4 with distilled water for a few minutes; agitate gently. Counterstain with light green (1% solution, 1 ml; dilute reagent, 100 ml). Rinse in dilute reagent. Mount in sirup to which a few drops of reagent are added. Bismuth stains orange-red.

Mercury, Silver, Gold

These metals occur in the tissues only if introduced artificially into the organism. They appear in the form of fine black or dark brownish granules. They all dissolve in a dilute solution of KCN. Hg and Ag dissolve in 15% HNO_3; Au does not. Hg and Au dissolve in bromine water or in Lugol's solution; Ag does not, although it will become bleached but still visible on account of the great density of AgBr and AgI in transmitted light.

For Au, the Elftman method (6) is recommended.

PROCEDURE

Treat paraffin sections for 24 hr to 6 days with 3% H_2O_2 at 37 C. Au salts are reduced to black or purple-brown metallic gold; most other pigments are completely bleached.

ORGANIC SUBSTANCES

Ascorbic Acid

Ascorbic acid reduces an acidified solution of $AgNO_3$ almost instantly; this reaction is utilized for its histochemical demonstration (11). On account of the pronounced diffusibility of ascorbic acid, localization is only approximate.

PROCEDURE

Place frozen sections of fresh unfixed tissue in a 1% solution of $AgNO_3$ in 3–5% acetic acid for about 3 min. Rinse in distilled water and fix in a 1–2% solution of Na thiosulfate for 1 min. Black granules indicate the presence of ascorbic acid.

Arginine

This amino acid can be demonstrated by various modifications of the Sakaguchi reaction; the simplest and probably the best is that of Baker (1), although even it is not absolutely dependable.

PROCEDURE

Any fixation is suitable. Use frozen or paraffin sections not thinner than 10μ. Protect the sections with a thin collodion membrane. Prepare the following reagent mixture: 1% solution of NaOH, 10 ml; 1% alpha naphthol in 70% alcohol, 0.5 ml; 1% solution of NaOCl (commercial Clorox diluted 1:5), 1 ml. Pour it immediately over the slide and leave it on for 15 min. Decant, blot the slide and mount it in a mixture of 3 parts of pyridine and 1 part of chloroform. Arginine is stained a rather pale reddish shade; the color is not very stable.

Uric Acid and Urates

Uric acid is the only known substance in alcohol-fixed tissue (besides melanin and possibly some of its precursors) which will reduce silver nitrate relatively rapidly, without any added extraneous reducer. This property can be utilized for its demonstration.

PROCEDURE

Run paraffin or celloidin sections of alcohol- or acetone-fixed tissues to alcohol. Transfer them directly into the following prewarmed mixture and keep them in it for 1–2 hr at 37–45 C, protected from light:

I. Silver-methenamine. To 100 ml of a 3% solution of methenamine add 5 ml of a 5% solution of $AgNO_3$. Shake until the initial heavy white precipitate disappears. Keep in the ice box.

II. 0.2 M borate buffer pH 8–8.4 (see under Buffers, p. 26).

For use, mix 25 ml of solution I, 20 ml of distilled water and 5 ml of the buffer.

Rinse sections in distilled water. Transfer them for 1 min (not longer) into a dilute (1–2%) solution of Na thiosulfate. Wash, counterstain as desired and mount. Uric acid and its salts are stained black. The crystalline structure of the deposits is fairly well preserved.

Adrenaline

Adrenaline is "chromaffin"; i.e., through the action of dichromates on adrenaline (and some other polyphenols) dark brownish substances (probably quinones) (10) are produced.

PROCEDURE

Fix thin slices of fresh tissue in Regaud's mixture (4 parts of 3–5% potassium dichromate, 1 part of 40% formaldehyde) for 24–48 hr. Cut frozen sections or embed tissue. In sections, the presence of brownish granules indicates the presence of adrenaline.

Secondary chromation of tissue fixed in dichromate-free mixtures is not possible.

Phenolic Substance of Enterochromaffin Cells

The enterochromaffin cells contain a derivative of resorcinol (15) which can be demonstrated either by its reducing power or by azo-coupling.

1. REDUCING POWER (SILVER TECHNIQUE)

Fix in Formalin or Bouin's solution. Treat the slides for 20 min to 2 hr with Gram-Lugol's iodine solution. Bleach with bisulfite. This treatment will cut down on the staining of the background. Wash under the tap and rinse in distilled water.

Prepare a silver-methenamine mixture (see above).

Incubate slides in the mixture for 12–24 hr at 37 C. Inspect them once in a while under the microscope; as soon as the enterochromaffin cells show up in a black shade, remove the slides and rinse in distilled water. If the background is too dark, differentiate carefully in an about 0.2% solution of ferric alum, containing about 0.5% of sulfuric acid, until the background is pale yellow. Rinse in distilled water. Tone in a 0.1–0.2% solution of gold chloride for 10 min. Fix for 1 min in a 1–2% solution of Na thiosulfate. Counterstain as desired. Enterochromaffin granules are dark purple-brown to black.

2. AZO-COUPLING REACTION

Dissolve about 50 mg of Naphthanil Diazo Red B (DuPont) or Red B Salt (National Aniline) or Fast Red Salt V (Ciba) in a few ml of distilled water; add a few drops or a saturated solution of borax. Pour the mixture over the slide; leave it on for 30–60 sec (not longer). Wash under the tap, counterstain with hematoxylin, dehydrate and mount. Enterochromaffin granules are an intense rusty red; the background is pale yellow.

Polysaccharides

The chemical identification of this group of substances depends on the oxidative liberation of aldehyde groups and their subsequent demonstration by Schiff's reagent (fuchsin sulfurous acid). The reaction does not distinguish the two most important representatives of this group, glycogen and the polysaccharide moiety of mucinoid substances. They can be differentiated by the amylase (diastase) test. If a section is incubated with a 1–2% dilution of extract of malt, U.S.P. (23), buffered around pH 7, for 1–2 hr at 37 C, glycogen and starch will go into solution, and any aldehyde reaction obtained after this treatment is due to other polysaccharides, mainly mucinoid substances, resistant to diastase. Slides should not be coated with collodion, and celloidin must be removed from celloidin-embedded material before the application of diastase because the enzyme will not diffuse through the membrane.

Two oxidizing substances can be used for the liberation of aldehyde groups, chromic acid (2) and periodic acid (20, 27). The former is milder and attacks mainly glycogen and mucin; polysaccharides of connective tissue (the nature of which is not well understood) are not visualized after its use, therefore it is much more selective for glycogen and mucin than periodic acid, which attacks all polysaccharides indiscriminately. The shade obtained after periodic acid is much more brilliant than that produced by chromic acid.

Solutions required are:

1. 0.2–0.5% solution of periodic acid.
2. 5% solution of chromic acid.
3. Schiff's reagent: Heat 1 g of basic fuchsin with 200 ml of water; when dissolved let it cool for 10 min; add about 2 g of sodium bisulfite and 1 ml of concentrated hydrochloric acid. Pour into a bottle, stopper it and allow to stand at room temperature until the shade turns into a straw yellow (usually 12–24 hr). Add about 0.5 g of charcoal, shake, let stand for a few minutes and filter. The filtrate should be colorless. Keep in the ice box.

Some batches of Schiff's reagent will keep unchanged for months; others will deteriorate in a few weeks, with or without assuming a pinkish shade. One may attempt to decolorize pink solutions by adding some more bisulfite to them; however, it is always advisable to test older solutions by staining a control slide, known to be positive for glycogen or mucin.

PROCEDURE

For *mucin*, any fixation will do; for *glycogen* use alcohol (80% or better) or Bouin's solution.

Run the slides to water. Immerse them either for 5 min in periodic acid or for 45–60 min in chromic acid. Wash under the tap for about 2 min.

Stain in Schiff's reagent diluted with an equal volume of distilled water for about 10 min. Little, if any, staining will be observed during this step, especially if chromic acid is used as pretreatment.

Rinse the slides. Immerse them for a few minutes in a 2–5% solution of sodium bisulfite. Wash under the tap for several minutes. It is during this step that staining becomes really apparent. Counterstain with hematoxylin, dehydrate and mount. Glycogen and mucin and, after periodic acid treatment, parts of the connective tissue, are stained intense purplish red.

Besides this chemical method, 3 more purely empiric staining methods are recommended, 1 for the identification of glycogen and 2 for mucin.

1. BEST'S CARMINE STAIN (3) FOR GLYCOGEN

Celloidin sections are preferable; paraffin sections should be protected by dipping them in dilute (0.5–1%) collodion in alcohol-ether between the 1st and 2d alcohol runs.

Stock solution is made by mixing carmine 2 g, potassium carbonate 1 g, potassium chloride 5 g and distilled water 60 ml and simmering gently for a few minutes; then cool. Add 20 ml of concentrated ammonia water. This solution will keep in the ice box for several months.

Stain the nuclei with hematoxylin; wash the slide. Stain for 5–10 min in the following mixture: stock solution, 10 ml; concentrated ammonia water, 15 ml; 95% alcohol, 15 ml. (This solution does not keep well and must be prepared fresh every time. It should be perfectly clear; if turbid, it must be filtered.) Differentiate in 60% alcohol to which a few drops of ammonia water are added. Dehydrate, clear and mount. Glycogen will be stained brilliant red. The stain is not absolutely specific; mucin, fibrin and mast cells may also be stained, although usually in a much lighter shade.

2. MAYER'S MUCICARMINE STAIN (28) FOR MUCIN

Stock solution is prepared by boiling 1 g of carmine with 0.5 g of aluminum chloride in 5–10 ml of water until the mixture becomes a deep but transparent red. Add, under continuous stirring, 100 ml of 50% alcohol. Allow the mixture to stand for 24 hr and filter.

Stain the nuclei with hematoxylin; wash the slide. Stain for 15 min to 2 hr either in the stock solution itself or in its dilution with up to 4 parts of 50% alcohol. Examine the section under the microscope at intervals; as soon as mucin stands out in a bright red shade, rinse the slide briefly in water, dehydrate and mount. Overstaining will result in a more or less intensely pink background.

3. METACHROMATIC STAINING OF MUCIN

Some of the thiazine and oxazine dyes stain various complex sulfuric esters of polysaccharides, such as chondroitin- and mucoitinsulfuric acids and heparin, in a metachromatic reddish shade (26) while the rest of the tissue is stained blue. This metachromasia is greatly weakened by subsequent alcohol treatment; therefore, if maximal contrast is desired the sections must be mounted in glycerin jelly. However, often a sufficient difference in shade will persist even after alcohol treatment.

Stain the section for about 20 min in a 0.05–0.1% solution of thionine or toluidine blue. Differentiate in 0.5–1% acetic acid until maximal contrast is obtained. Wash under the tap. Mount in glycerin jelly or dehydrate rapidly (!) in absolute alcohol, clear and mount.

Mucin, the ground substance of cartilage and granules of mast cells stain reddish purple, the background blue.

Nucleic Acids

Desoxyribose nucleic acids.—These can be demonstrated by Schiff's reagent after acid hydrolysis (8).

PROCEDURE

Use any fixation. Place the slides in N HCl preheated to 55–60 C for 5–10 min. Rinse in water. Transfer to Schiff's reagent (see p. 10) for 5–10 min. Rinse. Immerse in a 2–5% solution of Na bisulfite for 1 or 2 min. Wash under the tap. Desoxyribose nucleic acids are stained purple-red.

Ribose nucleic acids.—There is no simple specific stain known for ribose nucleic acids. They will stain with almost any basic stain; toluidine blue or pyronine are especially recommended. The basophilia is abolished by pretreatment with ribonuclease. The enzyme is available commercially; however, a sufficiently active and pure enzyme can be prepared in the laboratory.

To prepare ribonuclease (4), grind up beef or dog pancreas and let stand with about twice the volume of 1% acetic acid for 24 hr. Boil the mixture for 10 min (not longer), filter and neutralize with alkali to pH 7–7.4. Filter once more. Add a few crystals of camphor. Activity is preserved in the ice box for several months.

PROCEDURE

Alcohol or Formalin fixation is preferable. Do not protect the slides with collodion; in fact, remove collodion from celloidin-embedded tissues with alcohol-ether. Incubate the slides in the enzyme solution for 1–2 hr at 60–70 C. Incubate control slides with buffer of pH 7–7.4. Stain the slides with toluidine blue. In the enzyme-treated slides there is no basophilia due to ribose nucleic acid.

Lipids

The term lipid will be used here to include all kinds of substances occurring in the tissues which have solubility properties characteristic of neutral fats (soluble in alcohol, ether, acetone, chloroform, benzene, etc.; insoluble in water), regardless of their chemical structure. In the past, a number of allegedly specific methods have been used for the identification of certain types of lipids. However, it is known now that the specificity of these methods is highly doubtful (24). Only 3 types of lipid substances can be differentiated safely from the main body of lipids: cholesterol, plasmal and vitamin A.

General stains for lipids.—Dyes used for the demonstration of lipids must be oil-soluble; in fact, staining of fat by the methods outlined is a purely physical phenomenon; there is no chemical affinity between the dyes and the substance stained.

The simplest and best fixative is Formalin. Too long a sojourn (over 48 hr) in Formalin may render lipids less easily stainable.

There are a number of good fat stains such as Sudan III and Sudan IV, Oil Red B, Coccinel Red, Oil Blue N and Sudan Black B. Saturated solutions in 50–70% alcohol are used. Staining is faster with solutions of high alcoholic content, but there is a danger of loss of lipids from the tissue; solutions containing 50–55% alcohol are safe in the latter respect but stain much slower. It has been found lately that 1 of the best solvents for the dyes mentioned is 60% ethyl carbitol (Carbide & Carbon Chemicals Corp.). Staining is rapid and very little fat is removed.

For staining, transfer frozen sections to 50% alcohol for 1 min. Stain for 10 min to 3 hr, depending on the solution used. Rinse in 2 changes of 50% alcohol. Wash in water, counterstain as desired and mount in glycerol jelly.

CHOLESTEROL

1. SCHULTZ REACTION (34)

Immerse frozen sections in a 2.5% solution of iron alum (ferric ammonium sulfate $24H_2O$; violet crystals) for 24 hr at 37 C. Wash in distilled water, mount on slide and blot thoroughly.

Put a few drops of a mixture of equal volumes of concentrated sulfuric and acetic acids on the tissue and cover with a cover slip. Cholesterol and its esters stain deep blue or purplish; in a few minutes the shade will turn into a typical blue-green. Other shades than blue-green are not specific. Exact localization is usually impossible owing to the destructive action of the acid mixture.

2 DIGITONINE REACTION (38)

Immerse frozen sections for 4–6 hr in a 50% solution of digitonine in 50% alcohol. Rinse in 50% alcohol, followed by water. Mount in glycerin jelly. Under the polarizing microscope typical birefringent needles of the cholesterol-digitonine complex are seen. This reaction is specific for free cholesterol (and all 3-β-OH ketosteroids) and is not given by its esters.

VITAMIN A

This substance can be recognized by its fading green fluorescence. For technical details see Popper (33).

PLASMAL

Plasmal (7) is a mixture of long-chained (C_{16} and C_{18}) aliphatic aldehydes. It occurs in the tissues in the form of a cyclic glycerol-acetal (plasmalogen). On treatment with various hydrolyzing or oxidizing agents, plasmal is liberated and can be demonstrated by Schiff's reagent. However, it should be borne in mind that under certain conditions aldehydes can form from various unsaturated fats by oxidation (5); these aldehydes must be differentiated from plasmal by the use of adequate controls. Formaldehyde for fixation should be prepared with freshly boiled distilled water; frozen sections should be worked up promptly and not exposed to air longer than necessary.

PROCEDURE

Fix in Formalin for 24 hr. Cut frozen sections; store them in freshly boiled distilled water in a stoppered jar. Immerse sections in a 1% solution of $HgCl_2$ for 10 min. Rinse in distilled water. Transfer into Schiff's reagent (see p. 10) for 15 min. Rinse in water. Transfer into a 1–5% solution of sodium bisulfite for 2 min. Wash in several changes of water. Counterstain with hematoxylin.

Mount in glycerin jelly or, since the plasmal-fuchsin complex is lipid-insoluble, dehydrate in alcohols and mount in balsam. Plasmal will be stained purplish red.

Run a control section through the same routine except for omission of treatment with $HgCl_2$. Only the difference between the test section and the control is due to the presence of plasmal.

Oxidative Enzymes

OXIDASE

Synonyms for "oxidase" are indophenol oxidase, M nadi-oxidase, stable nadi-oxidase.

Although there can be no doubt that the formation of indophenol blue in the course of this "oxidase" reaction is due not to enzymatic activity but to the presence of fatty acid peroxides (25, 36) in the "active" structures, it will be described here because of reasons of tradition.

The following modification of Winkler-Schultze's method (35) is simple and does not necessitate the preparation of relatively large amounts of perishable reagents.

PROCEDURE

Dissolve (*a*) 20–50 mg of alpha naphthol in about 2 ml of 50%

alcohol; (*b*) 20–50 mg of p-aminodimethylaniline hydrochloride (Eastman #492) in about 2 ml of water. To a Coplin jarful of distilled water add 1 ml of strong ammonia water; pour in the reagents mentioned and stir the mixture.

Fix air-dried smears in a mixture for about 1 hr. Immerse slides in the mixture for 5 min, rinse and mount in glycerol. "Oxidase" granules are stained deep blue.

DOPA OXIDASE

This enzyme catalyzes the oxidation of dioxyphenylalanin (dopa) to melanin.

PROCEDURE (22)

Use unfixed tissue or fix thin slices in Formalin for 2 hr only; use frozen sections. Dissolve 100 mg of dioxyphenylalanin (Nutritional Biochemicals Corp., Cleveland) in 100 ml of water. To 3 parts of this solution add 1 part of phosphate buffer pH 7.3–7.4. Incubate tissues for 2–4 hr at 37 C. Wash, mount on slides, dehydrate, etc. Sites of activity are seen in shades of brown to black.

It is important to keep the pH of the solution between the limits specified. Below pH 7.2 the reaction is slowed considerably; above pH 7.5 there may be spontaneous oxidation of the substrate.

PEROXIDASE

Lison's zinc-leuco technique (ref. (24), p. 268) is so vastly superior to all other methods that it should be used exclusively. It is highly selective for hemoglobin.

PROCEDURE

Fix in Formalin or, even better, in Slonimski's mixture (Formalin with 2–3% potassium ferricyanide added). Paraffin or celloidin sections may be used, but frozen material gives distinctly better results.

Boil 1 g of acid fuchsin, acid violet or patent blue with 100 ml of 2% acetic acid and a few grams of zinc dust until the dye is decolorized. Add another 2–3 ml of acetic acid. The leuco-dye can be kept in the ice box for many months; in case of recolorization, boil once more until color is gone. For use, filter about 10 ml and add 2 ml of commercial (3%) H_2O_2.

Stain sections in the mixture for 2–5 min. Hemoglobin will be stained in the shade of the dye. Counterstain as desired. Dehydrate and mount in balsam.

Hydrolytic Enzymes

A number of hydrolytic enzymes are resistant enough to fixation and embedding to retain part of their activity, sufficient for histochemical demonstration.

In frozen material, alkaline and acid phosphatase and esterase can be demonstrated after short fixation (3–5 hr) in Formalin. For paraffin work, however, much better results are obtained after fixation in acetone. The latter fixative assures the best results with all hydrolytic enzymes, although for alkaline phosphatase and cholinesterase alcohol is entirely satisfactory.

Fixation and embedding.—The following schedule is suggested for all hydrolytic enzymes.

1. Fix thin slices of tissue in chilled (around 5 C) acetone for 12–24 hr.
2. Dehydrate in 2 more changes of acetone, 12 hr each.
3. Transfer to a mixture of equal parts of alcohol and ether for 6–12 hr.
4. Transfer to dilute (3–4%) collodion in alcohol-ether for 12 hr.

Steps (1) to (4) are preferably done at ice box temperature.

5. Drain and wipe pieces rapidly; transfer them to 2 changes of chloroform, 1 hr each.
6. Embed in paraffin at a temperature not exceeding 56 C. Use 3 changes. First change, 10 min, in an open dish. Second change, about 30 min, in a bottle with a perforated rubber stopper through which the bottle is connected (via the top ventilating hole of the oven) to a filter pump. The faucet is turned on slowly until sufficient vacuum is created to make the tissue bubble. A few minutes later the vacuum is carefully and gradually increased until the pump will run at full capacity. At no time should bubbling become so violent as to force paraffin into the tubing. A few minutes after bubbling has ceased the tissue is transferred to the 3d change, about 15 min, in an open dish. By this method most tissue blocks, unless excessively large, can be infiltrated with paraffin within 1 hr.

Sections should be cut about 3–6 μ and floated on warm (about 40–45 C) water. Thicker sections may not spread out entirely flat; in this case 15–20% acetone can be added to the warm water. Mounted sections should be dried at around 37 C and melted on the slide in the paraffin oven. Tissues will retain their activity both in paraffin blocks and in melted-on sections for many months and often years, whereas unmounted ribbons or mounted but unmelted sections may deteriorate in weeks.

The slides are run through xylol and alcohols to water. Often it is advisable to protect them with a thin layer of collodion to prevent the diffusion of the enzyme. For this purpose, after the 2d alcohol the slide is flooded with dilute (about 0.5%) collodion in alcohol-ether, the excess shaken off and the slide put back in the alcohol jar for about 1 min. In other cases, protection with collodion should be omitted.

Celloidin embedding is not recommended for enzyme work unless the blocks are cut and stained promptly. Dilute alcohol in which celloidin blocks are stored is harmful to enzymes.

ALKALINE PHOSPHATASE

This enzyme is rather resistant to various physical and chemical agents, and minor departures from the techniques suggested will not influence the results materially.

Two techniques are available and equally reliable. The older calcium-cobalt method (13) requires cheaper and easily available reagents; the newer naphthol technique (29) has the great advantage that the progress of the reaction can be observed directly.

1. CALCIUM-COBALT METHOD

Protection of the slide with collodion is advisable. Incubate in the following solution at 30–40 C for 1–12 hr (much longer incubation may produce various artifacts): 3% solution of Na glycerophosphate, about 5 ml; distilled water, about 40 ml; 2% solution of $CaCl_2$, about 5 ml; 20% solution of $MgCl_2$, a few drops; Na barbital powder, 1 knifepointful (0.3–1 g). Agitate the mixture until complete solution. In case long incubation is planned, add a few drops of 5% thymol or camphor in alcohol. The grade of glycerophosphate is not important; should it contain appreciable amounts of free phosphate, a precipitate will form which must be filtered off.

Wash slides under the tap. Immerse in a 1–3% solution of any soluble cobalt salt (chloride, nitrate, sulfate, acetate) for 5 min, then wash thoroughly under the tap. Immerse in a dilute solution of yellow ammonium sulfide (a few drops to a Coplin jarful of distilled water) for 1–2 min and wash under the tap. Counterstain as desired, dehydrate and mount. Sites of alkaline phosphatase activity are stained black or shades of brown-gray.

Possible sources of error are the presence of (1) preformed deposits of Ca phosphate which will stain black and (2) various dark pigments. To identify the former, run a control slide for Ca or remove deposits before incubation by treating the slide for a few minutes with a citrate buffer of pH 4.5–5. Pigments are visible in the unincubated slide; hemosiderin may be identified by the Prussian blue reaction (see p. 4).

2. NAPHTHOL METHOD

This depends on the hydrolysis of alpha-naphthyl phosphate in the presence of a diazonium compound. The naphthol liberated couples *in statu nascendi* with the diazonium compound to form brilliantly colored and highly insoluble azo dyes.

A number of "stable" diazonium compounds are on the market. In powder form they can be kept in the ice box for over a year without noticeable decomposition. Blue B Salt (DuPont) and Fast Blue Salt BN (General Dyestuff Corp.) are especially recommended. Chemically, both are tetrazotized dianisidine. They couple fast, and the azo dye formed is highly insoluble; in this way diffusion artifacts can be avoided, provided certain precautions are observed. In addition, the azo dye formed is insoluble in alcohol and xylol, thus permitting the mounting of the slides in balsam. However, it is soluble in a mixture of alcohol and xylol; for this reason, careful blotting of the slide between the last alcohol and xylol is most imperative. Almost all other azo dye products of both alpha and beta naphthol are xylol-soluble and require the mounting of slides in glycerin jelly.

It should be borne in mind that azo-coupling is not quite as fast a process as ionic reactions are. In case of highly active tissues the coupling reaction may lag behind the production of free naphthol; the latter may diffuse out from the sites of activity, and in this way some blurring of the picture may result, with secondary absorption of dye to neighboring structures. To prevent this, it is often advisable to let the enzymatic reaction take place under ice cooling or at a nonoptimal pH (between pH 7.6 and 8.4). In this way the rate of enzymatic hydrolysis is reduced while the rate of coupling is not noticeably affected; as a result, the picture obtained becomes sharper. Also, the diazo compound is rather unstable at higher temperatures and at elevated pH values; the decomposition products will stain the background in a brownish shade.

Protection with collodion is to be avoided; in fact, collodion should be removed with alcohol-ether from double-embedded tissues since the collodion membrane may stain deeply by the azo dye formed.

Incubate slides at 5–20 C, for 10 min or more in the following mixture: Dissolve 10–20 mg of Na alpha-naphthyl phosphate (Dajac Laboratories, 3430 W. Henderson St., Chicago 18) in about 50 ml of any buffer (barbital, borate, tris(hydroxymethyl)-amino-methane, etc.) of pH 7.6–8.4. Add about 30 mg of Blue B Salt dissolved in a few ml of water. The shade of the solution should be a light brown; if it turns bluish (presence of free naphthol), it should be filtered.

The reaction will usually become visible within less than 1 min. The slides may be inspected repeatedly under the microscope, and as soon as they appear dark enough, they are rinsed, counterstained as desired, dehydrated and mounted. Sites of enzymatic activity are a dusky purple-blue or blackish shade.

ACID PHOSPHATASE

This enzyme is sensitive to various chemicals and to heat. For fixation, only acetone is recommended. Infiltration with paraffin should not take longer than 1 hr. The composition of the incubating mixture should be followed closely. Even despite all precautions, false negative results are common. Two techniques are available, of which the old lead technique (19) is simpler and just as satisfactory as the newer naphthol method. The latter is not recommended.

PROCEDURE

Protection by collodion is unnecessary. Incubate slides in the substrate mixture for 1–12 hr at 37 C. Human prostate tissue requires only about 1 hr; other tissues 5–6 hr or more. At the end of the incubation, sites of activity appear chalky white.

The substrate mixture is composed of M acetate buffer pH 5 (30 ml of 6% acetic acid plus 70 ml of 13.6% $CH_3COONa{\cdot}3H_2O$), 2.5 ml; 5% lead nitrate, 1.0 ml; distilled water, 50.0 ml; 3% Na glycerophosphate, 5.0 ml. Any commercial brand of glycerophosphate containing about equal parts of the 2 isomers will do. The mixture will become quite turbid. Heat it to 50–60 C for about 5 min and filter into a Coplin jar.

Wash the slide thoroughly in distilled water, then for 1 min or so in 2% acetic acid and again in distilled water. Immerse in a dilute solution of yellow ammonium sulfide (a few drops to a Coplin jarful of distilled water) for 2–3 min. Wash under the tap. Counterstain as desired. Dehydrate in alcohols, clear in gasoline or tetrachloroethylene and mount in some synthetic resin (e.g., clarite) dissolved in the same solvents. Lead sulfide is somewhat soluble in xylene which will cause fading of the stain. Sites of acid phosphatase activity are stained in shades of brown-black.

Sources of error are the same as in the method for alkaline phosphatase (p. 17). In addition, with the technique for acid phosphatase, axons may become impregnated by lead in a nonenzymatic way. In case of doubt, inactivate a control slide by dipping it for 5 min either in Lugol's solution or in 0.25% HNO_3. Only the difference between the test slide and the control is due to enzymatic activity.

ESTERASE AND LIPASE

Lipases hydrolyze esters of carboxylic acids, especially the true fats, whereas esterases attack the simpler esters preferentially. However, there is a large group of enzymes of an intermediate type; they will hydrolyze almost any purely organic N-free ester substrate to some extent, although their substrates of preference may vary widely. A certain amount of differentiation is possible by using unsaturated substrates which are attacked by lipase only, or by adding bile salts to the substrate mixture. Cholates are inhibitors of esterases and activators of lipases.

Acetone fixation is preferable; alcohol may cause considerable inactivation.

There are 2 types of techniques available which yield similar results, differing only in minor but possibly important details of localization.

1. TWEEN TECHNIQUES (14, 18)

Tweens and related substrates are long-chained fatty acid esters of either sorbitan, the remaining hydroxyl groups of which are etherified with ethylene oxide chains of varying lengths, or of polyglycols. They are easily soluble in water. The following compounds, all made by the Atlas Powder Co., Wilmington, Del., are recommended as substrates: Tween 20 (laurate); Tween 40 (palmitate); Tween 60, G-9096-CJ and G-2151 (stearates); Tween 80 and G-2144 (oleates). Different batches of these compounds may vary considerably in their content of free fatty acid, which must be removed before they can be used as substrates. The best way to prepare ready-to-use stock solutions follows. Dissolve about 2% of the substance and 0.2% $CaCl_2$ in a 0.1M tris(hydroxymethyl) aminomethane-maleate buffer of pH 7.6. Add a few crystals of camphor and place the mixture in the incubator for 2 days. If it remains clear it can be used as is; if not, it must be filtered through a fritted glass or Seitz filter. It will keep in the refrigerator practically indefinitely.

The rate of hydrolysis decreases with increasing chain length; laurates are hydrolyzed at the fastest rate, stearates and oleates much slower; palmitates are in the middle. However, the crystal size of the precipitate also decreases with increasing chain length, and although laurates will give good reactions in 2–3 hr, Ca laurate forms crystals so coarse that finer localization is impossible. In most cases it pays to incubate for 12–24 hr with a stearate substrate and get a fine crystalline precipitate, permitting fairly exact localization.

Occasionally there is a severe patchy cytolysis in tissues incubated for over 8 hr, making the counterstaining of the slide practically impossible. This can sometimes be prevented by immersing the slides before incubation in 10% Formalin for about 10 min. There is no noticeable inactivation of esterase and lipase by this treatment.

For differentiation of esterase from lipase, add Na taurocholate in a concentration of about 0.005M (5 ml of a 2.5% solution to a Coplin jarful) to the substrate mixture. This will suppress esterase activity almost completely while the pancreatic type of enzyme (true lipase) is considerably activated.

Collodion protection is recommended only for saturated substrates; the unsaturated substrates will not diffuse through the membrane.

Incubate at 37 C for 12–24 hr in a mixture of 0.05M barbital or tris(hydroxymethyl)aminomethane-maleate buffer pH 7.2–7.6, 30–40 ml; 2% $CaCl_2$ solution, 2–3 ml; glycerol, 10–20 ml; substrate stock solution, 2–4 ml; camphor, a few small crystals. Wash under the tap; rinse in distilled water. Immerse in a 1–2% solution of lead nitrate for 15–20 min. Wash thoroughly in several changes of distilled water. Immerse in a dilute solution of yellow ammonium sulfide (a few drops to a Coplin jarful of distilled water) for 5 min. Wash under the tap. Counterstain as desired. Clear and mount as in the case of acid phosphatase (p. 19). Sites of esterase (lipase) activity are in shades of golden brown. Sources of error are the same as for the phosphatases.

2. NAPHTHOL ESTER TECHNIQUE (30)

This is based on the hydrolysis of naphthol acetate by esterase in the presence of diazonium salts. The topography of enzymatic activity is not exactly the same with esters of different naphtholic substances. However, since the only substrate readily available commercially is 1-naphthol acetate (Eastman #2380), the use of this ester is recommended. Other esters must be synthesized in the laboratory. Hydrolysis of naphthyl acetate is not noticeably influenced by bile salts.

Immerse slides at room temperature in a mixture prepared by blowing into 50 ml of 0.05M phosphate buffer, pH 6.3–7.0, with a pipet a solution of about 10 mg of 1-naphthol acetate in 1–2 ml of 60% acetone and stirring until the solution is clear. Dissolve about 30 mg of diazonium salt (see naphthol technique for alkaline phosphatase, p. 17) in a few ml of distilled water; pour it into the buffered substrate. If any turbidity results, the mixture must be cleared by rapid filtration or centrifugation. Sites of intense activity become colored in about 1 min; the reaction is complete in

10–20 min. By this time the mixture may become slightly turbid owing to decomposition of the diazonium salt. Wash under the tap. Counterstain as desired. Mount in glycerol-gelatin or balsam (see technique for alkaline phosphatase, p. 18).

CHOLINESTERASE

Enzymes hydrolyzing carboxylic acid esters of choline form a group the members of which have 1 important feature in common: they are all inhibited by very low concentrations (10^{-6} to 10^{-7}M) of eserine or Prostigmin. Otherwise, their substrate preferences and conditions of optimal activity may vary considerably. For histochemical purposes, long-chained fatty acid esters of choline are used (16). These esters are hydrolyzed by some choline esterases (both of the "nonspecific" and of the "true" type), and not by others.

Formerly the substrates were not available* and had to be synthesized in the laboratory. The synthesis of myristoyl choline will be given in detail; lauroyl and palmitoyl choline can be prepared in an analogous way.

Synthesis of myristoyl choline chloride. An equimolecular mixture of choline chloride and of myristoyl chloride (Eastman #2218; Matheson #5656) is heated to 130–140 C under constant stirring. In about 1 hr the evolution of HCl will cease and the mixture will solidify into a thick paste. The mass is washed with petroleum ether and subsequently extracted with boiling acetone (in the case of palmitoyl choline, about 5% alcohol should be added) and filtered while hot. On cooling (sometimes in the ice box), the ester chloride will precipitate in the form of a white crystalline solid. It is purified by 2 more recrystallizations from boiling acetone and dried in vacuo. It is stable in the ice box.

PROCEDURE

Prepare an approximately 0.02M (about 0.7%) stock solution of ester in warm (about 60 C) distilled water. Solution may require 1–2 hr. Add a crystal of camphor; store in the ice box. The solution may become somewhat opalescent.

Use alcohol or acetone fixation and protect slides with collodion. Incubate the slides for 4–48 hr (lauroyl choline, 4–6 hr; myristoyl choline, 8–24 hr; palmitoyl choline, 24–48 hr) in the following mixture: 0.05 tris(hydroxymethyl)aminomethane-maleate buffer pH 7.8–8, 45 ml; 0.1M (2.5%) cobaltous acetate, 5 ml; substrate stock solution, 1 ml; 0.1M (2%) manganese chloride, 2 drops. Wash under the tap.

---

* They are now available from Dajac Laboratories, 3430 W. Harrison St., Chicago 18.

Immerse the slides in a dilute solution of yellow ammonium sulfide (2 drops to a Coplin jarful of distilled water). Blackening of sites of enzymatic activity is quite gradual: for lauroyl choline, about 5 min; for myristoyl choline, 5–10 min; for palmitoyl choline, 15–30 min. Wash under the tap. Counterstain as desired, dehydrate and mount. Sites of enzymatic activity are stained black.

PHOSPHAMIDASE

This enzyme hydrolyzes the bond between P and N. The natural substrates are probably compounds like phosphoarginine and phosphocreatine; for histochemical purposes artificial synthetic substrates are used which are easier to prepare (17). Such substrates until recently were not available commercially.* The synthesis of 1 of them follows.

p-Chloroaniline-N-phosphonic acid.—Dissolve 26 g of p-chloroaniline in about 50 ml of hot (60–70 C) benzene. Cool the solution. Add, drop by drop, 10 ml of $POCl_3$. The hydrochloride of chloroaniline will separate almost immediately. Filter the mixture; pour 4–5 portions of hot benzene, about 10 ml each, over the precipitate, which is then discarded. To the filtrate add petroleum ether, drop by drop, until a distinct turbidity results. Place the mixture in the ice box, where a purplish gummy mass will separate. Decant the clear supernatant, evaporate it at 50–60 C under vacuum to about 30 ml, add 150–200 ml of petroleum ether and place the mixture in the ice box. The dichloride of the phosphonic acid will precipitate as white crystals. Decant and discard the supernatant; then wash crystalline mass twice with petroleum ether and dry it in vacuo. Dissolve it in an excess of ice-cold 5% ammonia water (about 50 ml for each gram of crystals); filter the solution. Add enough HCl to about pH 4–4.5. Place the mixture in the ice box; the free phosphonic acid will separate as a crystalline mass. Wash it 1st with a large amount of ice-cold distilled water, then with alcohol and ether and dry it in vacuo. Melting point, 156 C. Keep it in the ice box.

Prepare a 0.1M stock solution by dissolving 2.1 g in an ice-cold mixture of 50 ml of distilled water and 2 ml of strong ammonia under constant shaking. Solution is usually not complete. Filter the solution. Add a drop of phenolphthalein solution and titrate back with N acetic acid to a barely visible pink shade. Fill up to 100 ml. Add a few crystals of camphor. This stock solution will keep in the ice box for many months, probably indefinitely.

---

* They are now available from Dajac Laboratories, 3430 W. Harrison St., Chicago 18.

PROCEDURE

Do not protect slides with collodion; remove collodion from double-embedded tissues with alcohol-ether.

To 50 ml of 0.05 M maleate buffer pH 5.4–5.6 add 0.8 ml of a 5% solution of $Pb(NO_3)_2$; shake until the initial white precipitate disappears. Add 1 ml of substrate stock solution. Place the opalescent solution in the paraffin oven for about 10 min or until turbidity settles. Filter the solution into a Coplin jar. Incubate the slides at 37 C in the solution for 10–24 hr. Support the Coplin jar at an angle; place the slides with the tissue facing downward.

At the end of incubation remove the slides from the jar, wipe the precipitate from the back surfaces of the slides and rinse them in distilled water. Differentiate in a 0.1M citrate buffer pH 4.5 5 until the slide around the tissue has become completely clear. Rinse in water. Immerse in a dilute solution of yellow ammonium sulfide, counterstain and mount as in the method for acid phosphatase (p. 19). Sites of activity are stained brown to black.

Differentiation in citrate buffer is the most delicate step in the procedure. Underdifferentiation may leave large amounts of nonspecific precipitate in the tissue, and overdifferentiation may remove part or all of the product of enzymatic reaction.

The technique for phosphamidase in its present form is not completely dependable; false negative reactions and atypical localizations will be obtained once in a while despite all precautions.

BUFFERS FOR USE IN HISTOCHEMISTRY

The pH values in the tables which follow are only approximate but are sufficiently accurate for histochemical purposes.

0.2M CITRATE BUFFER

Solution *A:* 4.2 g of citric acid in 100 ml of water.

Solution *B:* 5.9 g of sodium citrate in 100 ml of water.

Add a few crystals of camphor to both solutions.

A, ml	*B*, ml	pH	*A*, ml	*B*, ml	pH
80	20	3.4	55	45	4.5
76	24	3.6	46	54	4.8
70	30	3.8	40	60	5.0
65	35	4.0	35	65	5.3
61	39	4.2	30	70	5.5

0.2M ACETATE BUFFER

Solution *A:* 1.2 ml of acetic acid in 100 ml of water.

Solution *B:* 2.7 g of Na acetate ($CH_3COONa \cdot 3H_2O$) in 100 ml of water.

Add a few crystals of camphor to both solutions.

A, ml	*B*, ml	pH	*A*, ml	*B*, ml	pH
87	13	3.8	40	60	4.8
80	20	4.0	30	70	5.0
73	27	4.2	21	79	5.2
62	38	4.4	14.5	85.5	5.4
51	49	4.6	11	89	5.6

0.2M MALEATE BUFFER

50 ml 0.4M acid Na maleate (2.32 g of maleic acid + 20 ml of N(4%) NaOH in 50 ml) + x ml of 4% NaOH diluted to a total of 100 ml.

ml NaOH	pH	ml NaOH	pH
0.5	4.6	5.8	5.6
1.0	4.8	7.6	5.8
1.8	5.0	10.0	6.0
2.8	5.2	12.5	6.2
4.0	5.4	14.5	6.4

Add a few crystals of camphor.

0.2M PHOSPHATE BUFFER

Solution *A:* monobasic Na phosphate ($NaH_2PO_4 \cdot H_2O$), 2.76 g in 100 ml.

Solution *B:* dibasic Na phosphate ($Na_2HPO_4 \cdot 7H_2O$), 5.36 g in 100 ml.

A, ml	*B*, ml	pH	*A*, ml	*B*, ml	pH
90	10	5.9	33	67	7.1
85	15	6.1	23	77	7.3
77	23	6.3	19	81	7.4
68	32	6.5	16	84	7.5
57	43	6.7	10	90	7.7
45	55	6.9			

0.2M TRIS(HYDROXYMETHYL)AMINOMETHANE-MALEATE BUFFER

Dissolve 29 g of maleic acid and 30.3 g of tris(hydroxymethyl)-aminomethane (Commercial Solvents Corp., New York 17) in 500 ml of distilled water. Add about 2 g of charcoal, shake, let stand for 10 min and filter. 40 ml of this stock solution + x ml of N (4%) NaOH diluted to a total of 100 ml.

ml NaOH	pH	ml NaOH	pH	ml NaOH	pH
9.0	5.8	16.5	6.6	22.5	7.6
10.5	6.0	18.0	6.8	24.2	7.8
13.0	6.2	19.0	7.0	26.0	8.0
15.0	6.4	20.0	7.2	29.0	8.2

Add a few crystals of camphor.

0.05M BARBITAL BUFFER

1.03 g of Na barbital in 50 ml + x ml of 0.1N HCl, diluted to a total volume of 100 ml.

HCl, ml	pH	HCl, ml	pH
5.0	8.8	26	7.8
7.5	8.6	31	7.6
11	8.4	36	7.4
15	8.2	41	7.2
19	8.0	43.5	7.0

Barbital buffers of higher molarity than 0.05M should not be used because barbituric acid will precipitate, especially in the lower ranges of pH.

0.2M 2-AMINO-2-METHYL-1,3-PROPANEDIOL BUFFER

2.1 g substance (Commercial Solvents Corp., New York 17) in 50 ml + x ml of N HCl, diluted to a total of 100 ml.

HCl, ml	pH	HCl, ml	pH
1.6	9.6	11.5	8.6
3.2	9.4	14.0	8.4
5.0	9.2	15.5	8.2
7.2	9.0	17.0	8.0
9.5	8.8		

0.2M BORATE BUFFER

Solution *A:* 1.24 g of boric acid in 100 ml.

Solution *B:* 1.9 g of borax ($Na_2B_4O_7 \cdot 10H_2O$) in 100 ml.

A, ml	*B*, m	pH	*A*, ml	*B*, ml	pH
90	10	7.4	55	45	8.4
85	15	7.6	45	55	8.6
80	20	7.8	30	70	8.8
70	30	8.0	20	80	9.0
65	35	8.2	10	90	9.2

REFERENCES

1. Baker, J. R.: The histochemical recognition of certain guanidine derivatives, Quart. J. Micr. Sc. 88: 115, 1947.
2. Bauer, H.: Mikroskopisch-chemischer Nachweis von Glykogen und einigen anderen Polysacchariden, Ztschr. f. mikr.-anat. Forsch. 33: 143, 1933.
3. Best, F.: Über Karminfärbung des Glykogens und der Kerne, Ztschr. wissensch. Mikr. 23: 319, 1906.
4. Brachet, J.: La détection histochimique des acides pentosenucléiques, Compt. rend. Soc. de biol. 133: 88, 1940.
5. Cain, A. J.: On the significance of the plasmal reaction, Quart. J. Micr. Sc. 90: 75, 1949.
6. Elftman, H., and Elftman, A. G.: Histological methods for the demonstration of gold in tissues, Stain Technol. 20: 59, 1945.
7. Feulgen, R., and Voit, K.: Über einen weitverbreiteten festen Aldehyd, Arch. f. d. ges. Physiol. 206: 389, 1924.
8. Feulgen, R.: Die Nuclealfärbung, in Abderhalden, E. (ed.): *Handbuch der biologischen Arbeitsmethoden* (Vienna: Urban & Schwarzenberg, 1926), p. 1053.
9. Frankenberger, Z.: Recherches histologiques sur la localisation du plomb, Compt. rend. A. d'anat. 16: 241, 1921.
10. Gerard, P.; Cordier, R., and Lison, L.: Sur la nature de la reaction chromaffine, Bull. d'histol. appliq. à la physiol. 7: 133, 1930.
11. Giroud, A., and Leblond, C. P.: Recherches histochimiques sur l'acide ascorbique ou vitamine C, Bull. d'histol. appliq. à la physiol. 11: 365, 1934.
12. Gomori, G.: Microtechnical demonstration of iron, Am. J. Path. 12: 655, 1936.
13. Gomori, G.: The distribution of phosphatase in normal organs and tissues, J. Cell. & Comp. Physiol. 17: 71, 1941.
14. Gomori, G.: The microtechnical demonstration of sites of lipase activity, Proc. Soc. Exper. Biol. & Med. 58: 362, 1945.
15. Gomori, G.: Chemical character of the enterochromaffin cells, Arch. Path. 45: 48, 1948.
16. Gomori, G.: Histochemical demonstration of sites of choline esterase activity, Proc. Soc. Exper. Biol. & Med. 68: 354, 1948.
17. Gomori, G.: Histochemical demonstration of sites of phosphamidase activity, Proc. Soc. Exper. Biol. & Med. 69: 407, 1948.
18. Gomori, G.: Histochemical localization of true lipase, Proc. Soc. Exper. Biol. & Med. 72: 697, 1949.
19. Gomori, G.: An improved histochemical technic for acid phosphatase, Stain Technol. 25: 81, 1950.
20. Hotchkiss, R. D.: A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations, Arch. Biochem. 16: 131, 1948.
21. Kóssa, J. v.: Über die im Organismus künstlich erzeugbaren Verkalkungen, Beitr. z. path. Anat. u. z. allg. Path. 29: 163, 1901.

22. Laidlaw, G. F.: The dopa-reaction in normal histology, Anat. Rec. 53: 399, 1932.
23. Lillie, R. D., and Greco, J.: Malt diastase and ptyalin in place of saliva in the identification of glycogen, Stain. Technol. 22: 67, 1947.
24. Lison, L.: *Histochimie animale* (Paris: Gauthier-Villars, 1936), pp. 189-209.
25. Lison, L.: Sur la recherche des oxydases par la réaction du bleu d'indophenol: Le cas des lipides, Bull. Soc. chim. biol. 18: 185, 1936.
26. Lison, L.: Une réaction micro et histochimique des esters sulfuriques complexes, la "réaction métachromatique," Bull. Soc. chim. biol. 18: 225, 1936.
27. McManus, J. F. A.: Histological demonstration of mucin after periodic acid, Nature, London 158: 202, 1946.
28. Mayer, P.: Über Schleimfärbung, Mitt. a. d. Zool. Stat. Neapel 12: 303, 1896.
29. Menten, M. L.; Junge, J., and Green, M. H.: A coupling histochemical azo dye test for alkaline phosphatase in the kidney, J. Biol. Chem. 153: 471, 1944.
30. Nachlas, M. M., and Seligman, A. M.: The histochemical demonstration of esterase, J. Nat. Cancer Inst. 9: 415, 1949.
31. Okamoto, K., and Utamura, M.: Biologische Untersuchungen des Kupfers: I. Histochemische Kupfernachweismethode, Acta scholae med. univ. imp. in Kioto 20: 573, 1938.
32. Okamoto, K.: Biologische Untersuchungen der Metalle: IV. Histochemischer Nachweis einiger metalle in den Geweben, besonders in Nieren u. deren Veränderungen, Acta scholae med. univ. imp. in Kioto 32: 99, 1942.
33. Popper, H.: Histologic distribution of vitamin A in human organs under normal and under pathologic conditions, Arch. Path. 31: 766, 1941.
34. Schultz, A.: Eine Methode des mikroskopischen Cholesterinnachweises am Gewebsschnitt, Zentralbl. f. allg. Path. u. path. Anat. 35: 314, 1924-25.
35. Schultze, W. H.: Zur Technik der Oxydasereaktion (Indophenolblausynthese), Zentralbl. f. allg. Path. u. path. Anat. 28: 8, 1917.
36. Sehrt, E.: Die histologische Darstellung der Lipoide der weissen Blutzellen etc., München. med. Wchnschr. 74: 139, 1927.
37. Wachstein, M., and Zak, F. G.: Bismuth pigmentation, Am. J. Path. 22: 603, 1946.
38. Windaus, A.: Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren Ztschr. f. physiol. Chem. 65: 110, 1910.

SECTION II

Fluid and Electrolyte Distribution

ASSOCIATE EDITOR—*Louis B. Flexner*

INTRODUCTION

THIS SECTION as originally planned was more comprehensive than it now stands. This is because of the 2 circumstances that pressure of other commitments prevented certain individuals from accepting an invitation to contribute and that the contributions which now appear utilize all the available space.

Perhaps the most notable omissions are the determination of circulating red cell mass, the chemical estimation of electrolytes in blood and tissue and the measurement of protein osmotic pressure. Determination of circulating red cell mass by radioiron has been described by Peacock *et al.* (4) and Gibson *et al.* (1); Kelly *et al.* (3) described and evaluated the simpler procedure in which radiophosphorus is substituted for radioiron. Reliable methods for chemical estimation of electrolytes in biologic material appear in several texts and in the literature. The Hepp osmometer for measurement of protein osmotic pressure is described in several places (2, 5, 6).

All of the contributions in this section have been submitted to authoritative reviewers for their suggestions. In all but one instance, comment has been of such kind that it was incorporated into the article by the author. I am sure that the authors join me in thanking the reviewers for their generous aid.

—LOUIS B. FLEXNER.

REFERENCES

1. Gibson, J. G., *et al.:* Measurement of the circulating red cell volume by means of two radioisotopes of iron, J. Clin. Investigation 25: 616, 1946.

2. Hepp, O.: Ein neues Onkometer, Ztschr. f. d. ges. exper. Med. 99: 709, 1936
3. Kelly, F. G., Simonsen, D. H., and Elman, R.: Blood volume determination in the human with red cells containing radioactive phosphorus, J Clin. Investigation 27: 795, 1948.
4. Peacock, W. C., *et al.:* The use of two radioisotopes of iron in tracer studies of erythrocytes, J. Clin. Investigation 25: 605, 1946.
5. Rehm, W. S.: An easily constructed Hepp osmometer, Science 100: 364, 1944.
6. Wagner, R. H.: Determination of Osmotic Pressure, in Weisberger, A. (ed.): *Physical Methods of Organic Chemistry* (New York: Interscience Publishers, Inc., 1949).

Measurement of Total Body Water

BERNARD B. BRODIE, *National Heart Institute, Bethesda, Md.*

A. USE OF ANTIPYRINE

METHODS FOR measuring the total body water of the living organism are usually based on the volume of distribution of a substance which, after intravenous injection, will distribute uniformly throughout all the body water. Sulfanilamide and thiourea have both been tried for this purpose (7, 14), but were unsatisfactory because of unequal distribution throughout the water of the body (15, 19, 20). Deuterium and tritium oxides are not subject to this criticism since presumably they are distributed throughout the body like ordinary water. They have been used to some extent in the measurement of body water (8, 9, 11, 13), but the complicated apparatus and techniques required for their estimation have prevented their general use.

Studies in the dog involving the distribution of antipyrine, a relatively nontoxic analgesic, demonstrated that it is distributed in tissues in proportion to their water content (3). The possibility was considered that antipyrine might be used to measure total body water of man in vivo. Subsequent exploration indicated that the substance is rapidly distributed in various human tissues in close proportion to their water content, is excreted in only negligible amounts and is metabolized in various individuals at rates ranging from 1 to 17%/hr and averaging 6%/hr (17). For a given individual, the disappearance rate is constant, thus allowing correction to be made by extrapolation of plasma levels to time of injection, as described later. A method for the estimation of antipyrine in plasma has been devised which is both simple and accurate (5).

The volumes of distribution of antipyrine and deuterium oxide were compared and found to agree well in normal subjects and in subjects with abnormal depots of fluid. Soberman (16) compared the volumes of distribution of antipyrine in rabbits with the total body water estimated by desiccation of the animals, with good agreement. Further confirmation of the validity of antipyrine measurements was obtained from calculation of the total body water from body specific gravity (2, 12). The values thus calculated agreed well with those obtained with the antipyrine method (1, 10).

Reproducibility of the antipyrine method for measuring body water was tested by repeating the measurement 4 times in each of 3 individuals at 48 hr intervals. Average deviation from the mean of the 4 measurements of each individual was 1.1, 1.8 and 1.5 l (3, 4.1 and 3.7% respectively), with maximal deviation of 3.1 l (18).

There are at least 2 errors in a procedure of this type: (1) that of chemical estimation, and (2) that involved in extrapolation of a line through several points to approximate the plasma concentration at zero time. With these errors, better reproducibility should not be expected. There is another possible error in that antipyrine is bound to plasma proteins to the extent of about 10% (17). However, the apparently uniform distribution of antipyrine between plasma and tissue water and the good agreement between total body water as measured by antipyrine, deuterium oxide, desiccation and body specific gravity suggest that there is compensatory binding in the tissues.

MEASUREMENT OF BODY WATER WITH ANTIPYRINE

This technique is used to measure body water in man.* A control sample of blood is drawn for estimation of a small blank value which is used for correcting subsequent samples. An exact amount of antipyrine,† about 1 Gm in 50 ml of 5% glucose solution, is injected intravenously from a buret or calibrated syringe over about 5 min. The middle of the injection period is taken as zero time. Blood samples are subsequently withdrawn at approximately 2, $3^1/_2$ and 5 hr and the exact times noted. Plasma or serum is separated by centrifugation and stored in stoppered tubes for subsequent analysis. Antipyrine is stable in plasma for at least several days when kept at refrigerator temperature.

In subjects with edema or with abnormal depots of fluid, the blood samples are withdrawn at 5, 7 and 9 hr after administration of antipyrine. The samples are taken at longer intervals after injection than in normal subjects because the time for uniform distribution of the compound is longer under these conditions. The body water is calculated as:

---

* Antipyrine has been used by Soberman to measure total body water in rabbits (16). Its rate of metabolic transformation is relatively rapid in this species (about 40%/hr) but is constant for a given animal. Uniform distribution throughout the body is quickly established so that blood samples may be drawn at relatively short intervals following the injection. One hundred mg/kg of antipyrine is administered and blood samples are drawn 50, 75 and 100 min later. Measurements with antipyrine have not been reported in the dog. Despite the rapid rate of metabolic transformation of antipyrine in dogs (about 50%/hr) it is probable that here, too, suitable measurements of body water can be made by using large doses of the compound.

† Both antipyrine (USP) and NAAP may be sterilized and made pyrogen-free by passing 20% solutions, under pressure, through an asbestos filter pad. Eli Lilly & Company, Indianapolis 6, Ind., will supply investigators with ampules of sterile antipyrine in 20% solution. Winthrop-Stearns, Inc., will supply NAAP in crystalline form. For further information, contact Special Chemical Division, Winthrop-Stearns, Inc., 1450 Broadway, New York.

$$\text{body water (l)} = \frac{\text{amt. of drug injected (mg)}}{\text{conc. in plasma water at 0 time (mg/l)}}$$

Plasma concentration at zero time is the concentration which would have resulted at the time of injection if uniform distribution had been instantaneous and if none of the substance had been metabolized or excreted. The zero time concentration is calculated by plotting the plasma levels of antipyrine on the log scale of semilogarithmic paper as a function of time on the arithmetical scale and projecting the line drawn through the points back to the time of injection. The 3 points representing the plasma levels should fall on a straight line.

The concentration of antipyrine in plasma water is calculated by dividing the plasma level of antipyrine at zero time by 0.93.

MEASUREMENT OF ANTIPYRINE IN PLASMA

In this method, antipyrine is estimated in the filtrate after deproteinization of plasma with zinc hydroxide. Sodium nitrite is added and the concentration of the 4-nitrosoantipyrine that is formed measured at 350 mμ in a Beckman ultraviolet spectrophotometer. The method is specific in that it excludes from the measurement any metabolic derivatives of antipyrine. There is a small plasma blank which is equivalent to about 1 μg of antipyrine/ml. The following drugs have been tested for their interference in the procedure: acetophenetidin, aspirin, caffeine, codeine, morphine, atropine, sulfanilamide, sulfadiazine, phenobarbital, penicillin G, pentobarbital, acetanilid, Pyramidon and Demerol. Of these, only sulfadiazine interferes with the antipyrine procedure.

a) *Reagents.*—1. Zinc reagent: 100 g of $ZnSO_4 \cdot 7H_2O$ and 40 ml of 6N sulfuric acid are dissolved in water and diluted to 1 l.

2. 0.75N sodium hydroxide.
3. 4N sulfuric acid.
4. 0.2% sodium nitrite solution.

b) *Technique.*—To 2 ml of plasma‡ in a 50 ml Erlenmeyer flask add 2 ml of water and 2 ml of zinc reagent. Add 2 ml of 0.75N sodium hydroxide drop by drop, with continuous swirling of the flask, and shake for an additional 1/2 min. After 10 min, centrifuge at about 3500 rpm for 15 min. Transfer 3 ml of the clear supernatant fluid to a Corex or quartz cuvet and add 1 drop of 4N sulfuric acid. Read the optical density at 350 mμ. Three ml of water plus 1 drop of 4N sulfuric acid is used for the zero setting. Add 2 drops of 0.2% sodium nitrite solution to both the unknown and the zero setting and read the optical density again after 20 min.§ Subtract from this the optical density obtained before the addition of the nitrite.‖ Finally, subtract the small blank value obtained from blank plasma.

---

‡ Although not tried in this laboratory, the procedure can probably be carried out with considerably smaller plasma samples. To measure the optical densities of small volumes, microcuvets holding 50-100 cu mm, which require a special cuvet carrier, and a microdiaphragm attachment for the Beckman spectrophotometer can be obtained from Pyrocell Manufacturing Co., 207 E. 84th St., New York.

c) *Standard curve.*—Standards are prepared by placing 3 ml of a known solution of antipyrine (10–20 μg/ml) in 0.07N sulfuric acid into a Corex or quartz cuvet and reading the optical density before and after the addition of sodium nitrite. As the zero setting, 0.07N sulfuric acid similarly treated is used. The optical densities are proportional to concentration. A concentration of 10γ/ml of antipyrine gives an optical density of about 0.350. Standards are run with each set of determinations, since there is a daily variation of about 3% in optical density.

Antipyrine added to plasma in amounts from 20 to 50 μg/ml has been recovered with an average error of ±2.5% (165 recoveries).

B. USE OF N-ACETYL 4-AMINOANTIPYRINE (NAAP)

During studies of the metabolism of Pyramidon, 1 of its metabolic products, N-acetyl 4-aminoantipyrine (NAAP), was found to distribute in dog tissues in proportion to the water and to be negligibly bound onto plasma proteins (4). A colorimetric method for its estimation has been devised so that an ultraviolet spectrophotometer is not required.

In normal man, NAAP disappears from the body in different persons at the uniform rate of approximately 8%/hr; the major part of the substance is eliminated by the kidneys and only a minor part is metabolized. The time for uniform distribution is somewhat longer than that of antipyrine; hence it is preferable to use antipyrine for subjects with abnormal fluid depots. Since NAAP is almost quantitatively eliminated in human urine, the possibility of calculating the volume of distribution of the compound on the basis of its urinary excretion and a single plasma level was considered. This procedure possesses 2 inherent sources of error: (1) the small amount of NAAP metabolized in the body (0–15 mg of a 1 g dose in 3 hr), and (2) the amount of NAAP which is concentrated (50–70 times) in the urine and left in the ureter and pelves even though the bladder is completely emptied. These errors are in the direction of making the estimate of body water too large. The magnitude of the combined errors was assessed by comparing the volume of dilution of NAAP as measured from a single plasma level and urinary secretion with that calculated by extrapolation of a line through 3 plasma concentrations (as with antipyrine). The comparisons for 30 subjects were in fairly good agreement, those obtained by the urine collection procedure averaging 1 l higher than those obtained by the plasma extrapolation technique (6). These results suggest that although the errors of the urine collection procedure are in the expected direction, they are not large. However, the urine collection procedure cannot be utilized in dogs or in other animals that metabolize appreciable amounts of the substance.

---

§ Optical density reaches a maximum in about 20 min and fades slowly after 35 min.

‖ The small optical density of the solution before addition of the nitrite is not changed by addition of the nitrite. It is therefore corrected for by subtracting its reading from the final reading.

The volume of distribution of NAAP has not been compared with that of deuterium oxide. However, the volumes of distribution of NAAP (using the extrapolation technique) and of antipyrine have been compared in normal human beings with satisfactory agreement, antipyrine space averaging 1.8 l higher than NAAP in 12 observations. Measurements of body water, using NAAP, have been repeated 4 times, in each of 3 individuals, at 48 hr intervals. Average deviation from the mean of the 4 measurements of each individual was 1.2, 1.5 and 1.8 l (3, 4.4 and 6.4% respectively), with maximal deviation of 3.4 l (6).

MEASUREMENT OF BODY WATER WITH NAAP

Two techniques may be employed for measuring body water in man[¶] with NAAP.[**] The first, which depends on extrapolation of a line through 3 plasma concentrations to approximate the plasma concentration at zero time, is similar to that already described for antipyrine. Since the drug takes longer to distribute throughout the body water than does antipyrine, the blood samples are withdrawn at 3, $4^1/_2$ and 6 hr following the injection.

The second technique involves the measurement of a single plasma level and the measurement of urinary excretion 3 hr following administration of NAAP. The body water is then calculated as

$$\text{Body water (l)} = \frac{\text{amt. of drug injected (mg)} - \text{amt. excreted (mg)}}{\text{conc. in plasma water (mg/l)}}$$

[¶] In dogs, NAAP disappears at a rate averaging only about 15%/hr, in contrast to antipyrine which disappears about 3 times as rapidly. In the use of antipyrine to measure body water in the dog, large doses are required to insure sufficiently high concentration of compound in the last plasma sample for accurate estimation. The relatively slow disappearance of NAAP permits use of considerably smaller doses.

[**] See footnote (*) on page 32.

ESTIMATION OF NAAP IN PLASMA AND URINE

NAAP is estimated in the filtrate after deproteinization of plasma with zinc hydroxide. The urine, after suitable dilution (generally 1:50), is handled in the same way as the plasma. The NAAP is hydrolyzed by acidifying and heating the filtrate and the resulting 4-aminoantipyrine is diazotized and coupled with alpha naphthol. The dye formed is assayed at 490 mμ in a spectrophotometer or photoelectric colorimeter. The method is specific in that it excludes from the measurement any metabolic derivatives of NAAP. There is a small plasma blank which is equivalent to about 1 μg of NAAP/ml. The following compounds have been tested for their interferences in the procedure: acetophenetidin, aspirin, caffeine, codeine, atropine, sulfanilamide, sulfadiazine, phenobarbital, penicillin G, pentobarbital, acetanilid, Pyramidon, Demerol and p-aminohippuric acid. Since acetophenetidin, p-aminohippuric acid, acetanilid, Pyramidon, sulfanilamide and sulfadiazine were found to interfere in the procedure, these drugs should not be administered to subjects during or before the test period.

a) *Reagents.*—1. Standard NAAP solution, 100 mg/l; 100 mg of NAAP is dissolved in water and diluted to 1 l. Working standards are made by diluting the stock solution with water.

2. Zinc reagent: 100 mg of $ZnSO_4 \cdot 7H_2O$ and 40 ml of 6N sulfuric acid are dissolved in water and diluted to 1 l.

3. 2.5N hydrochloric acid.

4. 0.2% sodium nitrite.

5. 1% ammonium sulfamate.

6. 4N sodium hydroxide.

7. 5% alpha naphthol. Resublimed alpha naphthol[††] is dissolved in absolute alcohol just before use.

b) *Technique.*—To 2 ml of plasma[‡‡] or suitably diluted urine (containing 20–60 μg of NAAP) in a 50 ml Erlenmeyer flask add 3 ml of water and 2 ml of zinc reagent. Add 2 ml of 0.75N sodium hydroxide drop by drop with continuous swirling of the flask, then shake for an additional 1/2 min. After 10 min, transfer to a test tube and centrifuge at about 3500 rpm for 15 min. Transfer 4 ml of the supernatant fluid to a colorimeter tube and add 1 ml of 2.5N hydrochloric acid. Cover the tube with a glass marble to prevent evaporation and heat in a boiling water bath for 30 min. Cool, add 0.5 ml of sodium nitrite and place in refrigerator for 10 min.

[††] Alpha naphthol is conveniently sublimed as follows. The substance is placed in an evaporating dish over which is placed a glass funnel whose diameter is a little less than that of the dish. The dish is heated gently with a small flame on a sand bath, and the alpha naphthol which condenses on the sides of the funnel is periodically scraped away with a spatula. It is stored in a dark bottle.

[‡‡] See footnote (‡) on page 33.

Add 0.5 ml of ammonium sulfamate, shake and wait 3 min. Then add 0.1 ml of alpha naphthol solution, followed by 1 ml of 4N sodium hydroxide. Let stand for 5 min, then read at 490 mμ in a spectrophotometer or photoelectric colorimeter. A reagent blank run through the procedure is used for the zero setting.

c) *Standards.*—These are prepared by taking 4 ml of a known solution of NAAP (5–10 μg/ml), adding acid and hydrolyzing and colorizing as with the unknowns. Standards should be run concurrently with the unknowns since there are slight daily variations in color intensity.

NAAP added to plasma in amounts from 15 to 30 μg/ml may be recovered with an average error of ±2% (125 recoveries).

Comment by Nello Pace

The introduction of antipyrine and NAAP as agents for measurement of total body water is most welcome as a simplified technique for clinical use. The validity of the results has been checked in an unusually careful and thorough fashion by the New York University group, and the method appears to give consistent values over a wide variety of applications. However, as pointed out by the originators of the method, there are certain drawbacks that should not be lost sight of in applications of the method, so that the search for better substances continues. These drawbacks include a fairly rapid rate of metabolism of the material in the body and a selective localization of the antipyrine on the plasma protein. The former is not so serious, but the latter requires the assumption of compensatory binding by tissue proteins to explain the close agreement obtained between antipyrine space and true water space. It is always undesirable, although it may sometimes be unavoidable, to depend on compensating errors for measurements, because in some conditions the errors may change unpredictably.

It is somewhat puzzling that antipyrine is not excreted more rapidly by the kidneys. If it penetrates all the water spaces of the body as rapidly and readily as does labeled water, and it seems to on the basis of the equilibration time reported, it would be expected to appear in the glomerular filtrate in the same concentration as in the plasma and to appear in the urine at a 20- to 30-fold higher concentration. Since this does not seem to occur, one must postulate either a unique impermeability of the glomerular membrane alone for antipyrine or a high order of tubular resorption. Either of these would be of considerable interest. NAAP seems to follow a much more expected type of behavior in this regard.

A point to be emphasized is that of waiting at least 2 hr after injection before taking the 1st plasma sample in normal individuals, and longer in patients. Recent unpublished data on detailed mixing curves of tritium-labeled water injected intravenously reveals that about 1/3 of the mixing of the labeled water occurs with a half-time of 35–40 min in young normal individuals and much more slowly in various types of patients. It is imperative to wait until the mixing process is complete before beginning the measurements to be used for extrapolating the disappearance rate, or falsely low values for total body water are obtained.

Without detraction from the antipyrine methods, which appear exceedingly practicable for many clinical applications, it is still well to remember that to be certain of measuring the true water space of the body accurately, nothing succeeds like labeled water.

REFERENCES

1. Behnke, A. R., Jr., and Osserman, E. F.: Personal communication.
2. Behnke, A. R., Jr.; Feen, B. G., and Welham, W. C.: J.A.M.A. 118: 495, 1942.
3. Brodie, B. B., and Axelrod, J.: J. Pharmacol. & Exper. Therap. 98: 97, 1950.
4. Brodie, B. B., and Axelrod, J.: J. Pharmacol. & Exper. Therap., 99: 171, 1950.
5. Brodie, B. B., *et al.:* J. Biol. Chem. 179: 25, 1949.
6. Brodie, B. B.; Berger, E. Y.; Axelrod, J.; Dunning, M. L.; Porosowska, Y., and Steele, J. M.: Unpublished observations.
7. Danowski, T. S.: J. Biol. Chem. 152: 207, 1944.
8. Flexner, L. B.; Gellhorn, A., and Merrell, M.: J. Biol. Chem. 134: 35, 1942.
9. Hevesy, G., and Hofer, E.: Nature, London 134: 879, 1934.
10. Messinger, W. J., and Steele, J. M.: Proc. Soc. Exper. Biol. & Med. 70: 316, 1949.
11. Moore, F. D.: Science 104: 157, 1946.
12. Pace, N., and Rathbun, C.: J. Biol. Chem. 158: 685, 1945.
13. Pace, N., *et al.:* J. Biol. Chem. 168: 459, 1947.
14. Painter, E. E.: Am. J. Physiol. 129: 744, 1940.
15. Sise, H. S.: Proc. Soc. Exper. Biol. & Med. 40: 451, 1939.
16. Soberman, R.: Proc. Scc. Exper. Biol. & Med. 71: 172, 1949.
17. Soberman, R., *et al.:* J. Biol. Chem. 179: 31, 1949.
18. Steele, J. M., *et al.:* Am. J. Physiol., 162: 313, 1950.
19. Waterhouse, A., and Shannon, J. A.: Proc. Soc. Exper. Biol. & Med. 50: 189, 1942.
20. Williams, R. H., and Kay, G. A.: Am. J. Physiol. 143: 715, 1945.

Estimation of Plasma Volume by Dye Dilution Method

FRANCIS P. CHINARD,* *Johns Hopkins University School of Medicine*

The general principle for estimation of plasma volume depends on: a known amount of some foreign substance, such as a dye, injected into the blood stream, and a sample of blood taken after sufficient time has elapsed to insure uniform distribution (i.e., complete mixing) in the plasma. From the concentration of the substance in this sample and the amount injected, the distribution volume of the injected substance can be calculated. If the injected substance leaves the blood in significant amount during the period between injection and sampling, the rate of disappearance must be determined. This rate can be established by successive samplings of blood. From a time-concentration curve of the data it is possible to calculate by extrapolation the concentration of the substance that would have obtained had there been no loss from plasma. It is essential that a substance be used that does not leave the blood stream too rapidly; otherwise the rate of disappearance cannot be calculated with sufficient accuracy. A substance which leaves the blood at a reasonably slow rate (about 5%/hr) is the blue dye T-1824, first investigated by Dawson, Evans and Whipple (8) and later adopted for estimation of plasma volume by Gregersen, Gibson and their collaborators (see (11) for references). The dye is easily synthesized (15) and is available commercially.† T-1824 is preferred to red dyes such as brilliant vital red because presence of small amounts of hemoglobin in the plasma or serum will not cause serious error in determination of the concentration of T-1824. Its slow rate of disappearance is presumably due to its preferential binding to the albumin fraction of the plasma proteins, as demonstrated by Rawson (26). The fact that the dye does leave the plasma so slowly permits, in many cases, the use of the concentration of the dye 10 min after injection for calculation of distribution volume of the dye. The equivalence of this concentration at 10 min to the concentration calculated by extrapolation has been extensively investigated and validated by Noble and Gregersen (22). Exceptions are detailed below. Other extrapolations and formulations for the dye disappearance curve have been suggested to describe periods up to 24 hr after injection. There appears to be little justification in their use for estimation of plasma volume.

---

* Scholar in the Medical Sciences of the John and Mary R. Markle Foundation The previously unpublished experiments reported here were for the most part carried out at the Hospital of the Rockefeller Institute for Medical Research, New York, in the department of Dr. D. D. Van Slyke, to whom the author is deeply indebted for his encouragement.

PROCEDURE‡

a) Injection of dye.—Dosage should be approximately 1.0 ml of 0.5% solution l of expected plasma volume in order to insure adequate optical density in the plasma samples. Expected plasma volume can be calculated from Gregersen's average value of 45 ml of plasma/kg of body weight (11). Because of the opacity of the dye solution it is not possible to use the calibrations of the injection syringe. Several techniques have been suggested for injection of a known amount of dye.

---

† From William R. Warner & Co., Inc., 113 W. 18th St., New York 11, N. Y.

‡ Plasma volume estimated by the T-1824 dilution method may vary considerably in 24 hr with variations in physical activity, eating, etc. Further, if the subject has had a meal within a few hours of the test the plasma may be quite turbid; this will introduce an error if an extraction method is not used for determination of the dye. For this reason it is recommended that subjects fast for 6 hr and rest in bed for at least 1/2 hr before the dye is injected. This is obviously impossible if repeated estimations of plasma volume are to be made over a 24 hr or longer period. The acetone extraction method is recommended for these special circumstances.

1. WASH-OUT TECHNIQUE.—The dye solution is drawn into the injection syringe through a filler needle in an amount proximate to that desired. The filler needle is removed, air bubbles are forced out and the solution is brought exactly to the end of the syringe needle adapter; a sterile sponge is used to wipe off any excess dye. A pencil mark is placed on the plunger just at the point where it emerges from the barrel. The syringe is adapted to a needle inserted in a vein of the recipient; the hub of the needle must be filled with blood. The dye is injected and blood drawn back into the syringe 3 times, with the plunger drawn back to the pencil mark each time. Syringe and needle are then removed together. The amount of dye injected is found by calibrating the syringe by weight, with dye or with a solution of a standardized acid. It is essential that the plunger not be rotated during injection of the dye or during calibration, else an error of several per cent may be introduced in the amount of dye delivered. This wash-out technique may be difficult to use if the patient's veins tend to collapse. Calibration must correct for the dead space of the needle.

2. SINGLE INJECTION TECHNIQUE.—A holder such as the Krogh-Keys syringe pipet§ is very helpful. The volume to be delivered is set as in method (1); emptying of the syringe is accomplished by a single stroke. No wash-out is necessary, but the dead space of the needle must be corrected for, or included in the calibration. This technique is particularly suitable for children and other uncooperative patients.

3. INJECTION OF PREVIOUSLY DETERMINED AMOUNT.—With the device described by Barnett and Fellers (1) it is possible to inject quite accurately a known amount of dye. Calibration of the syringe or correction for dead space of the needle is not necessary. The dye is pipetted accurately from a sterilized pipet into an upper cup (a syringe barrel may be used), then drawn into a syringe through a 3-way stopcock. The syringe should be wet with 5% glucose or 0.85% NaCl before the dye is drawn into it. Then, several volumes of glucose or NaCl solution are admitted to wash out the upper container and syringe.

The chief disadvantage of the technique is that 1 min or more may be required to complete delivery of the dye into the blood stream.

b) Sampling.—Blood should be collected without stasis. Serum or plasma from heparinized blood may be used. Use of oxalate as an anticoagulant is inadvisable because of the danger of hemolysis. The following samples are required: (*a*) blank immediately before injection of the dye; (*b*) 10 min sample, or (*c*) samples at approximately 20, 30, 40 and 50 min after injection. The volume required for each sample depends on the size of the cuvets to be used in measurement of the optical densities. In cases of shock and cardiac failure it is necessary to use the value for the dye concentration found by extrapolation because mixing does not appear to be even nearly complete within 10 min (22). In normal individuals and when the plasma volume is changing rapidly, the 10 min sample may be used.

---

§ Obtainable from Macalaster Bicknell Company, Cambridge, Mass.

c) *Determination of dye concentration in clear samples.*—Two aliquots of blank plasma and aliquots of the dye-stained plasma samples are accurately pipetted into photometer cuvets. To 1 cuvet containing blank plasma[§] is added a known amount of dye in a measured volume of 0.85% NaCl.[¶] The amount of dye to be added to bring the optical density of this, the standard, close to the optical densities of the unknowns is calculated as follows:

Let V'_p = expected plasma volume (see above)
A = µg of dye injected
a = µg of dye to be added per ml of blank plasma

Then

$$a = \frac{A}{V'_p}$$

To each of the other cuvets is added a volume of 0.85% NaCl equal to that in which the amount of dye a was added to prepare the standard. The cuvets are rotated to insure complete mixing of the contents. Optical densities of the contents are read in a photoelectric spectrophotometer at $\lambda = 620$ mµ with water or air for the zero setting. From the optical densities of each sample containing dye, including the standard, the optical density of the blank sample is subtracted; net optical density due to the dye is thereby obtained. From the net optical density of the standard and the amount of dye added a point is plotted on paper with Cartesian co-ordinates relating optical density and dye concentration per ml of plasma. A straight line is drawn through this point and the origin. This is the standard curve; from this, the concentration of the dye in each of the other samples is estimated. When the highest accuracy is desired, several standards should be used so that their net optical densities will bracket the optical densities of the unknowns. The standard curve deviates from the Bouguer-Beer law at high concentrations of T-1824.

[§] The extinction coefficient of T-1824 is decreased by about 10% in plasma as compared with an aqueous solution, presumably because of the binding to albumin. The standard must be made in plasma and optimally in the patient's own plasma.

[¶] T-1824 is not stable indefinitely in 0.85% NaCl. The solution should be made at most a few hours before use (13).

d) Determination of dye concentration in turbid samples.—The simple procedure just outlined cannot be used for such turbid plasma samples as are encountered in patients with diabetic acidosis or the nephrotic syndrome. Because optical density of such turbid plasma samples may vary significantly with slight changes in temperature, with time or with slight differences in the handling of the samples, attempts to determine the concentration of T-1824 in such samples without further treatment are to be deprecated. This is because the apparent extinction coefficient of the dye varies with the degree of turbidity (4, 9). A standard curve in the patient's own plasma is not satisfactory because turbidity may vary considerably during an experiment. Several procedures have been suggested to circumvent this difficulty (4, 14, 18, 25). The simplest—and one which can be used with the Coleman Model 6A Clinical spectrophotometer, for example—follows.

ACETONE EXTRACTION METHOD (4).—Two 1.0 ml aliquots of blank plasma and 1.0 ml aliquots of dye-stained plasma are accurately pipetted into 12 × 75 mm Pyrex test tubes.* To each of the dye-stained and 1 of the blank plasma samples is added exactly 0.2 ml of 0.85% NaCl. To the remaining blank sample is added a known amount of the dye (as in the procedure for clear plasma) but contained in 0.2 ml of 0.85% NaCl; this is the standard. The contents of the tubes are mixed. To each tube is then added 3.0 ml of reagent grade acetone from a buret with a stopcock lubricated with a Silicone grease. Ordinary stopcock grease must not be used. Each tube is stoppered immediately after addition of the acetone and shaken vigorously for 10 sec. The stoppers are removed and the tubes covered with Parafilm† and centrifuged for 10 min at a relative centrifugal force (RCF) of approximately 1200 times gravity. The supernatant solutions (which contain most of the dye) are transferred to 12 × 75 cm photometer cuvets. The cuvets are sealed with Parafilm to prevent evaporation of acetone and centrifuged for 10 min at RCF = 1200 to remove any suspended protein particles or air bubbles adhering to the walls. Optical densities are read at $\lambda = 620$ mμ; concentrations are calculated as for clear plasma (paragraph *c*). Optical densities of the blanks vary between 0.000 and 0.003 for plasmas whose optical densities before extraction with acetone may have been as high as

---

* If larger or smaller amounts of plasma are used, all additions must be changed proportionately.

† Available through E. Machlett & Son, 220 E. 23d St., New York 10, N. Y.

1.75. The fraction of the dye extracted by the acetone from the protein is a function of the protein concentration; for this reason, if the protein concentration is changing rapidly during an experiment, it is necessary to establish a standard curve for every unknown sample obtained, as is done for the standard.

e) Correction for hemolysis.—Hemoglobin may be present in the samples available for analysis, because of hemolysis, in amounts detectable by simple visual inspection of the plasma; if this occurs a correction is necessary. This is difficult, if not impossible, because hemoglobin may exist not only as hemoglobin but as oxyhemoglobin, carboxy hemoglobin, methemoglobin and occasionally as sulfhemoglobin in different proportions in different samples. The absorption spectra are different for each and it would be necessary to know the concentrations of each to make an accurate correction.‡ This difficulty is avoided if the acetone extraction method is used, for it is not affected by the degree of hemolysis that one is likely to encounter if the precautions mentioned earlier for drawing blood samples are observed.

f) Correction for residual dye from previous injections.—It is recommended that the acetone extraction procedure be used when dye is present from earlier injections. The optical density of unstained plasma, even from patients with high icteric indexes, is so small compared with that due to the dye that it may be ignored without introducing a significant error. If the acetone method is not used, this blank optical density must be considered; it is then necessary to assume that the blank optical density has not changed over periods of hours or even days. (The decade photometer devised by Nickerson (21) may be used instead of a spectrophotometer. Gregersen has given a detailed description of the procedure (11) and has found no difficulty in correcting for residual dye with this instrument.)

g) Calculation of plasma volume.—1. FROM CONCENTRATION AT 10 MIN.—Let A be the amount of dye injected and C_o the concen-

---

‡ An approximate correction can be made as follows. Let D represent optical density of a sample at wavelength indicated by the subscript. Find $\frac{D'_{620}}{D'_{550}} = K'$ for a sample of plasma containing "hemoglobin" but no dye. Find $\frac{D''_{620}}{D''_{620}} = K''$ for a sample containing dye but no "hemoglobin." Find D_{620} and D_{550} for the sample containing both dye and "hemoglobin." Then the density due to dye alone in that sample at λ = 620 mμ is

$$D_{\mathrm{T-1824}} = \frac{D_{620} - K'D_{550}}{1 - \frac{K'}{K''}}$$

This expression presupposes that we are dealing with a single species of hemoglobin in all the samples.

tration of dye in plasma calculated from the standard curve. Plasma volume is:

$$V_p = \frac{A}{C_o}$$

2. BY EXTRAPOLATION. The data are plotted on semilog paper, with concentrations on the logarithmic scale and time of sampling on the linear scale. The best-fitting straight line that can be obtained is drawn through the points and extended to the time of injection. The concentration found at this intercept is used to calculate the plasma volume as above.

3. CORRECTION FOR RESIDUAL DYE. If the blank sample contains dye from previous injections, the concentration of the dye in the blank sample (i.e., the sample drawn just before injection of the dye) is subtracted from the concentration obtained by extrapolation. This difference is used to calculate plasma volume.

h) *Calculation of whole blood and red cell volumes from plasma volume and hematocrit.*—Let V_{wb} be the whole blood volume, V_{rbc} red cell volume, V_p plasma volume, Ht hematocrit reading for blood sampled just before injection, and k_{ht} a constant to correct the hematocrit for plasma trapped between the cells. Then

$$V_{wb} = \frac{100 \times V_p}{100 - k_{ht} \times Ht}$$

and

$$V_{rbc} = V_{wb} - V_p$$

Different values for k_{ht} are used by different investigators; most use 0.94 or 0.95. This calculation contains the implied assumption that the hematocrit reading of blood drawn from a vein is identical with that of blood anywhere in the vascular system. This assumption is known to be false. Accordingly, the calculated whole blood and red cell volumes will be in error by the extent that the hematocrit reading for venous blood differs from the true average blood hematocrit reading. There is, for example, an apparent loss of red cell mass in patients with the nephrotic syndrome following intravenous administration of human serum albumin where the red cell mass is calculated from the hematocrit and plasma volume (5, 17). Calculations of fluid shifts based on changes of hematocrit values are likely to be seriously in error; such calculations should be avoided. When possible, red cell mass should be estimated by one of the direct methods (e.g., 10, 16, 20, 23, 28, 29).

i) *"Normal" values.*—Tabulations of plasma and whole blood volumes by the T-1824 method for normal man and experimental animals are given in Reeve's review (27). For man, plasma volume averages about 45 ml/kg and whole blood volume (calculated from plasma volume and corrected hematocrit reading) about 80 ml/kg. For infants and children, the most useful data are in the publications of Russell (30), Morse *et al.* (19) and Brines *et al.* (2).

j) Reproducibility of T-1824 dilution volumes.—In 14 patients, sets of determinations of T-1824 dilution volumes were carried out to obtain some idea of the reproducibility and of the over-all error of the whole procedure (5). In some patients as many as 4 injections of dye were made in 1 hr; dilution volumes were calculated from the dye concentrations at 10 min in all cases. No corrections were made for fluid shifts. The means and percentage deviations from these means were calculated for each set; the coefficient of variation of these percentage deviations was ±1.6. Hence the maximal error in estimating the dilution volume of the dye from concentration in plasma at 10 min was of the order of 3.5%. This is not necessarily a measure of the error in estimation of the plasma volume.

COMMENT

The considerable controversy (e.g., (24)) regarding the validity of the methods used for estimation of plasma volume necessitates a somewhat more complete discussion than would otherwise have been required. Limitations of space preclude as elaborate a discussion as that given by Reeve (27). It is hoped that what follows may be of use in tempering both the enthusiasm of the proponents and the criticism of the opponents.

Shortly after injection of the dye into 1 vein, the plasma concentration in blood sampled from another vein shows an abrupt rise to a peak, followed by an almost equally abrupt drop and then a much more gradual fall. Much of the general controversy centers about the significance of these various portions of the time-concentration curve. Some authors have suggested that when the peak concentration is reached, "mixing" is complete; the blood should be sampled at that time and the plasma volume calculated from the dye concentration found at that time. Others have suggested that the rapidly descending portion of the curve should be used to extrapolate back to the time of injection. Both groups consider the rapidly descending portion of the curve to represent loss of dye from the circulation rather than mixing. The majority, however, extrapolate back to the time of injection from the more slowly descending portion of the curve; they consider that the concentration of dye in the plasma of blood sampled 10 min after injection of the dye gives an adequate approximation to this extrapolation in most cases. The contention of the first 2 groups concerning the significance of the peak concentration as well as of the rapidly descending portion of the curve is seriously weakened by the fact that the distribution volume of the dye calculated from the concentration at 10 min or by extrapolation is independent of the rate of injection of the dye. For example, in 1 patient 2 injections of dye were given, the first in 20 sec, the second, of the same amount of dye, in 165 sec. There was a sharply defined peak following the first injection, but no such sharp peak following the second slower injection, nor was there a rapidly descending portion of the curve. The plasma volumes calculated from the concentration estimated by extrapolation back to the time of the injection from the slowly descending portion of each curve and the plasma volumes calculated from the dye concentration at 10 min after each injection did not differ by more than 2% (5). It appears that the peak concentration and the slope of the rapidly descending portion of the curve may be determined largely by the rate of injection of the dye.

Further objections to use of T-1824 for estimation of plasma volume have been based (*a*) on the fact that dye injected in the blood stream appears in the thoracic duct lymph within about 10 min and (*b*) on the possibility that large amounts of the dye are rapidly removed by the reticuloendothelial system. It appears that the *amount* of dye in the lymph is generally small compared with the *amount* injected, although the *concentration* may approach that in plasma (6); the objection is valid qualitatively. Quantitatively, it may not be valid. With regard to the possible role of the reticuloendothelial system, the following observations are pertinent. Repeated injections of identical and different amounts of dye at identical and different rates of injection in the same individual result in nearly identical distribution volumes of the dye following each of the several injections (3, 5). Injections of dye bound to human serum albumin and injections of aqueous solutions of the dye also result in nearly identical distribution volumes for the dye. Injections of an aqueous solution of the dye and of the dye *chemically* coupled to human serum albumin in experimental animals also result in nearly identical distribution volumes for the dye (5). Finally, the distribution volumes of several large proteins have been reported to be not significantly different from the distribution volume of the dye in experimental animals (12). The differences found in the distribution volumes are within the overall limits of the experimental error for the whole procedure. The concept that the reticuloendothelial system plays a significant and unpredictable role in the disappearance of the dye from the circulation during the first 10 min following the injection of the dye is not compatible with these results. Crispell, Porter and Nieset (7) have reported on the distribution volumes at 10 min of T-1824 and human serum albumin tagged with I^{131}. The means for the 24 cases compared checked well, but there was as much as 10% difference in several of the individual comparisons. The agreement between these independent methods appears to be less satisfactory than that for the T-1824 method with itself.

Conclusion.—The T-1824 dilution method, when properly used, gives consistent and reproducible results. That the dilution volume of the dye is actually the plasma volume has not been conclusively demonstrated. Aside from this, the chief drawback to use of T-1824 is the unpleasant color that the dye imparts to light-skinned individuals and may take weeks or months to disappear. It is recommended that patients, relatives and surgeons be informed that T-1824 has been injected and that the evidence may persist for some time; it will eventually disappear.

NOTE.—This section was reviewed by M. I. Gregersen.

REFERENCES

1. Barnett, H. L., and Fellers, F. K.: A simple quantitative method for intravenous injection of small volumes of fluid, Science 106: 401, 1947.
2. Brines, J. K.; Gibson, J. G., and Kunkel, P.: The blood volume in normal infants and children, J. Pediat. 18: 447, 1941.
3. Campbell, W. N.; Sokalchuk, A., and Penman, R.: Validity of T-1824 in plasma volume determination in the human, Am. J. Physiol. 152: 563, 1948.
4. Chinard, F. P., and Eder, H. A.: The determination of concentration of the dye T-1824 in normal and lipemic plasmas, J. Exper. Med. 87: 473, 1948.
5. Chinard, F. P.; Lauson, H. D., and Eder, H. A.: To be published.
6. Courtice, F. C.: The blood volume of normal animals, J. Physiol. 102: 290, 1943.
7. Crispell, K. R.; Porter, B., and Nieset, R. T.: Studies on plasma volume using human serum albumin tagged with radioactive iodine^{131}, J. Clin. Investigation 29: 513, 1950.
8. Dawson, A. B.; Evans, H. M., and Whipple, G. H.: Blood volume studies: III. Behavior of a large series of dyes introduced into the circulating blood, Am. J. Physiol. 51: 232, 1920.
9. Gibson, J. G., 2d: Quoted by Thorn, G. W., *et al.:* J. Clin. Investigation 24: 802, 1945.
10. Gibson, J. G., 2d, *et al.:* The measurement of circulating red cell volume by means of two radioactive isotopes of iron, J. Clin. Investigation 25: 616, 1946.
11. Gregersen, M. I.: A practical method for determination of blood volume with the dye T-1824, J. Lab. & Clin. Med. 29: 1266, 1944.
12. Gregersen, M. I.; Boyden, A. A., and Allison, J. B.: Comparison of plasma volume measured with the blue dye T-1824 and with beef albumin, beef globulin and the pneumococcus polysaccharide SIII, Federation Proc. 4: 27, 1945.

13. Gregersen, M. I., and Stewart, J. D.: Simultaneous determination of plasma volume with T-1824 and the "available fluid" volume with sodium thiocyanate, Am. J. Physiol. 125: 142, 1939.
14. Harington, C. R.; Pochin, E. L., and Squire, J. R.: A simplification of the Evans blue method of blood volume determination, Clin. Sc. 4: 311, 1940.
15. Hartwell, J. L., and Fieser, L. F.: Coupling of *o*-tolidine and Chicago acid, Org. Synth. 26: 12, 1936.
16. Kelly, F. J.; Simonsen, D. H., and Elman, R.: Blood volume determination in the human with red cells containing radioactive phosphorus (P^{32}) and with pure human albumin, J. Clin. Investigation 27: 795, 1948.
17. Luetscher, J. A., Jr.: The effect of a single injection of concentrated human serum albumin on circulating proteins and proteinuria in nephrosis, J. Clin. Investigation 23: 365, 1944.
18. Morris, C. J. O. R.: Chromatographic determination of Evans blue in plasma and serum, Biochem. J. 38: 203, 1944.
19. Morse, M.; Cassels, D. E., and Schlutz, F. W.: Blood volumes of normal children, Am. J. Physiol. 151: 448, 1947.
20. Nachman, H. M., *et al.:* A comparative study of red cell volumes in human subjects with radioactive phosphorus tagged red cells and T-1824 dye, J. Clin. Investigation 29: 258, 1950.
21. Nickerson, J. L.: A decade photometer for determination of blood volume with the dye T-1824, Rev. Scient. Instruments 15: 69, 1944.
22. Noble, R. P., and Gregersen, M. I.: Blood volume in clinical shock. I. Mixing time and disappearance rate of T-1824 in normal subjects and in patients in shock; determination of plasma volume in man from 10 minute sample, J. Clin. Investigation 25: 158, 1946.
23. Peacock, W. C., *et al.:* The use of two radioactive isotopes of iron in tracer studies of erythrocytes, J. Clin. Investigation 25: 605, 1946.
24. Peters, J. P.: Role of sodium in the production of edema, New England J. Med. 239: 427, 1948.
25. Phillips, R. A.: A method for determination of the blue dye T-1824 in plasma, J. Exper. Med. 77: 421, 1943.
26. Rawson, R. A.: The binding of T-1824 and structurally related diazo dyes by the plasma proteins, Am. J. Physiol. 138: 708, 1943.
27. Reeve, E. B.: Methods of estimating plasma and total red cell volume, Nutrition Abstr. & Rev. 17: 811, 1947–48.
28. Reeve, E. B., and Veall, N.: A simplified method for determination of circulating red-cell volume with radioactive phosphorus, J. Physiol. 108: 12, 1949.
29. Reid, A. F., and Orr, M. K.: A rapid method for determining blood volumes by use of P^{32}-labeled red cells, J. Clin. Investigation 29: 313, 1950.
30. Russell, S. J. M.: Blood volume studies in healthy children, Arch. Dis. Child. 24: 88, 1949.

Determination of Thiocyanate Space

HOWARD A. EDER, *Cornell University Medical College*

Since the report of Crandall and Anderson in 1934 (1), the volume of distribution of injected thiocyanate (the thiocyanate space) has been used as a measure of the volume of extracellular fluid in the body. Thiocyanate has the desirable characteristics of ease of determination and slow excretion which make determination of its space relatively simple.

The validity of the use of thiocyanate space as a measure of the volume of extracellular fluid depends on being able to substitute in this equation:

$$\text{extracellular fluid vol} = \frac{\text{mass of SCN in extracellular fluid}}{\text{conc. of SCN in extracellular fluid}}$$

The numerator must be calculated by subtracting the amount excreted from the amount injected. This requires that none be formed or destroyed in the body and that the amount excreted can be determined. Elkinton and Taffel (2) showed in the dog and Meister and Freis (7) in man that an injected dose of thiocyanate can be completely recovered in the urine. Furthermore, the thiocyanate remaining in the body must all be confined to the extracellular fluid. Crandall and Anderson, however, showed thiocyanate to be present in the red cells and Kaltreider *et al.* (6) found it in gastric juice and saliva in concentrations 7–12 times that in the serum. This means that the amount injected minus the amount excreted is greater than the amount distributed only in the extracellular fluid, making the numerator erroneously high. The magnitude of this error depends on the total amount of thiocyanate present in extracellular sites, and this in practice cannot be determined.

To determine the thiocyanate concentration in the extracellular fluid (the denominator), all portions of the extracellular fluid must have the same concentration as the sample taken. This requires, first, that sufficient time be allowed for the thiocyanate to pass into all parts of the extracellular fluid and to reach equilibrium. When the plasma concentration of thiocyanate is plotted against time on a semilog plot (Fig. 1), a curve with 2 components is obtained. The first part, *AB*, with a steadily decreasing slope probably represents the period of transfer of thiocyanate from plasma to extravascular sites. The second portion, *BC*, has a constant slope proportional to the rate of urinary excretion of thiocyanate. At any point on the curve beyond *B* it is probable that the plasma and extracellular fluid have reached equilibrium. In normal man this occurs anywhere between ½ and 4 hr. In the edematous subject this takes much longer and in some cases may take as long as 12 hr (5). If the sample is taken at any point on *AB* the denominator will be erroneously large and the calculated space too low.

Second, since plasma is used ordinarily as the sample of extracellular fluid, the thiocyanate concentration in the plasma at equilibrium must be identical with that in the extravascular extracellular fluid. That this is not the case has long been known (8). Recently Scatchard *et al.* (9) showed that plasma albumin binds thiocyanate, and Scheinberg and Kowalski (10) derived equations to correct for this binding at varying concentrations of thiocyanate and albumin. These corrections show that the concentration of free thiocyanate in the plasma is appreciably lower than the total concentration. At low total thiocyanate concentrations the free thiocyanate may constitute only half the total. At low plasma albumin concentration the error from protein binding in the plasma is markedly reduced. Albumin may be present in the extravascular fluid in appreciable amounts although at low concentrations, and failure to correct for binding by this protein can also cause error. Unless the total amount of this protein is known, no correction for this can be made, and correcting only for plasma albumin binding will erroneously increase the apparent volume of distribution. Failure to correct for binding by plasma albumin makes the denominator erroneously high. Since the numerator is also erroneously high, these errors tend to cancel each other to an unknown extent.

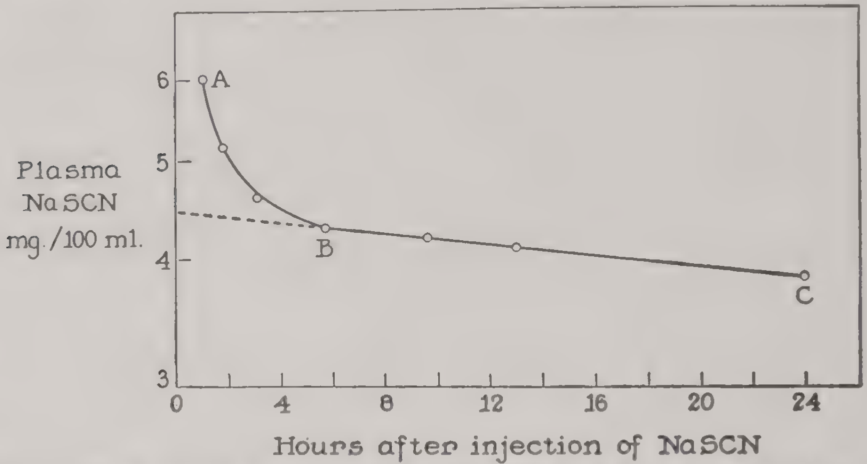

FIG. 1.—Total plasma concentrations of NaSCN in first 24 hr after injection of 1 g in a slightly edematous adult weighing 70 kg.

Because of these numerous uncontrolled variables, it can be expected that the volume of distribution of thiocyanate will differ from the volume of distribution of other substances also distributed predominantly in the extracellular fluid. Thus in 8 dogs Gaudino *et al.* (4) found the average inulin space to be 21.7% of body weight compared with a mean thiocyanate space of 31.3. In 5 of the dogs the mean sodium space was 30.5% compared with the thiocyanate space of 31.1% for the group. It should be emphasized that in an individual subject there may be considerable distortion of the relationship between 2 spaces when the relative magnitude of the variables changes: thus in the 8 dogs the ratio of inulin space to thiocyanate space ranged from 0.64 to 0.83 due primarily to variations in the thiocyanate space. In man Kaltreider *et al.* (6) found the mean sodium space to be 21.1% of body weight as compared with the thiocyanate space of 23.5%. In dogs Winkler *et al.* (11) found chloride, sodium and thiocyanate distributed through approximately 25, 28 and 36% of body weight respectively. Of special significance in this report is the fact that whereas the absolute spaces differed, when the extracellular fluid volumes of these dogs were altered the absolute *changes* in volume of distribution were of the same magnitude for chloride, sodium and thiocyanate. All these observations indicate that the determination of thiocyanate space has some value as an empiric method for estimating the volume of extracellular fluid and can be used for approximating changes in this volume. Until the variables previously described can be controlled it is not permissible to use thiocyanate as a true measure of extracellular fluid space.

PROCEDURE

a) *Determination of thiocyanate in plasma.*—1. REAGENTS.—(*a*) Standard sodium thiocyanate solutions: From reagent grade sodium thiocyanate prepare solutions containing 2, 5, 10 and 15 mg/100 ml. These solutions are stable and keep well at room temperature.

(*b*) Ferric nitrate: Add 50 g of reagent grade $Fe(NO_3)_3 \cdot 9H_2O$ and 25 ml of reagent grade nitric acid to a 1000 ml volumetric flask and fill to the mark with distilled water.

(*c*) 10% trichloracetic acid: Keep this solution in the cold when not in use.

2. TECHNIQUE. Pipet 10 ml of 10% trichloracetic acid into a 15 ml centrifuge tube, add 1 ml of heparinized plasma or serum (oxalate and citrate interfere with the determination). Stopper and shake well. After 10 min, centrifuge at 2500 rpm for 10 min. If particles of protein are floating in the supernatant, filter the supernatant through cotton. Transfer 4 ml of the supernatant to a 15 × 150 mm cuvet. Add 3 ml of the ferric nitrate reagent and mix well. The color will fade conspicuously on exposure to sunlight, so this step and all subsequent steps should be carried out in artificial light. The tubes are covered with a dark cloth and allowed to stand for 15 min. They are read in a Coleman Junior spectrophotometer at 460 mμ. The standard curve is obtained by adding 1 ml of each of the 4 standard solutions to 10 ml portions of 10% trichloracetic acid. The color is then developed as with the plasma

LIBRARY

supernatant. The reagent blank is determined by adding 3 ml of ferric nitrate reagent to 4 ml of the 10% trichloracetic acid.

The concentration-optical density curve is plotted from the optical density of the standard solutions after subtracting the reagent blank. The optical density of the plasma blank is subtracted from the optical density of the thiocyanate containing plasma and the concentration of sodium thiocyanate is then read directly from the standard curve.

b) Determination of space.—Five per cent sodium thiocyanate† is injected intravenously. The dose is that estimated for each subject to result in an equilibrium plasma concentration of between 5 and 10 mg/100 ml. For the nonedematous individual this will require injection of 0.3–0.5 ml/kg of body weight and for an edematous patient, as much as 1.0 ml/kg. Before the injection, blood should be taken for the blank determination. The solution is injected from a calibrated syringe at a rate no faster than 10 ml/min. (Patients occasionally complain of transient headache or dizziness, especially when injection is made too fast.) Blood samples are drawn at 3, 8 and 12 hr after injection and, in edematous subjects, also at 24 hr.

c) Calculation.—The plasma concentrations of sodium thiocyanate are plotted against time, as in Figure 1. The straight portion of the curve *BC* is then extrapolated back to zero time and the concentration read from the intercept at this point. This procedure eliminates determining thiocyanate in the urine, which is a procedure beset with difficulties. The equation used for calculation of thiocyanate space is:

$$\text{Thiocyanate space (l)} = \frac{\text{mg NaSCN injected} \times 10}{\text{mg NaSCN/100 ml plasma}}$$

As previously indicated, this equation has only empiric usefulness and makes no correction for thiocyanate present in other than extracellular fluid or for protein binding.

Normal values with this method range from 25 to 35% of body weight. In lean individuals the space is relatively higher than in obese subjects. In children, spaces tend to be higher than in adults and in infants may run to as high as 45% of the body weight (3). In patients with edema the space is increased, and when diuresis occurs there is usually good correlation between weight loss and decrease in thiocyanate space.

NOTE.—This section was reviewed by M. I. Gregersen.

---

† Sterile ampules containing 20 ml of 5% solution are obtainable from Wm. R. Warner and Company, New York City.

REFERENCES

1. Crandall, L. W., Jr., and Anderson, M. X.: Estimation of the state of hydration of the body by the amount of water available for the solution of sodium thiocyanate, Am. J. Digest. Dis. 1: 126–131, 1934.
2. Elkinton, J. R., and Taffel, M.: The apparent volume of distribution of sulfocyanate and of sulfanilamide in the dog, Am. J. Physiol. 138: 126–135, 1942.
3. Fellers, F. X., *et al.:* Change in thiocyanate and sodium spaces during growth, Pediatrics 3: 622–623, 1949.
4. Gaudino, M.; Schwartz, I. L., and Levitt, M. F.: Inulin volume of distribution as a measure of extracellular fluid in dog and man, Proc. Soc. Exper. Biol. & Med. 68: 507–511, 1948.
5. Gilligan, R. D., and Altschule, M. D.: The rate of attainment of diffusion equilibrium of thiocyanate between plasma and transudates following intravenous injection of sodium thiocyanate in patients with edema, J. Clin. Investigation 18: 501–506, 1939.
6. Kaltreider, N. L., *et al.:* Determination of the volume of extracellular fluid of the body with radioactive sodium, J. Exper. Med. 74: 569–590, 1941.
7. Meister, F. C., and Freis, E. D.: The metabolism of thiocyanate after prolonged administration in man, Am. J. M. Sc. 218: 549–555, 1949.
8. Rosenbaum, J. D., and Lavietes, P. H.: Lipoid-thiocyanate in serum, J. Biol. Chem. 131: 663–674, 1939.
9. Scatchard, G.; Scheinberg, I. H., and Armstrong, S. H., Jr.: Physical chemistry of protein solutions: V. The combination of human serum albumin with the thiocyanate ion, J. Am. Chem. Soc. 72: 540–546, 1950.
10. Scheinberg, I. H., and Kowalski, H. J.: The binding of thiocyanate to albumin in normal human serum and defibrinated blood with reference to determination of thiocyanate space, J. Clin. Investigation 29: 475–482, 1950.
11. Winkler, A. W.; Elkinton, J. R., and Eisenman, A. J.: Comparison of sulfocyanate with radioactive chloride and sodium in measurement of extracellular fluid, Am. J. Physiol. 139: 239–246, 1943.

Radioisotope Method for Determining Volume of Extracellular Fluid in the Animal Body

J. F. MANERY, *University of Toronto*

General evaluation.—Anatomically, the extracellular fluids of the body include the water of the blood and lymph, interstitial fluids of tissues, pleural, pericardial, peritoneal and synovial fluids, cerebrospinal fluid, eye humours and glandular secretions. Physiologically, the term extracellular fluid is applied only to those liquids into which nonmetabolized substances freely diffuse from the plasma, and since cerebrospinal fluid, eye humours and glandular secretions do not fulfil this criterion, they are not included in the usual measurements of extracellular water.

Like other methods of measurement, the radioisotope procedure depends on the dilution which a known amount of the isotope or other substance undergoes when it is injected into the body. The value obtained is often designated as the volume of distribution of the substance injected. Ideally, such a chemical should possess the following attributes: (1) it must be readily soluble in water and insoluble in fats; (2) it should be nontoxic in amounts sufficiently large for accurate determination; (3) its concentration in extracellular water should be predictable from its concentration in plasma; (4) it must not be excreted in appreciable amounts during the experiment; (5) it must not participate in metabolic processes which will affect its detection, and (6) it should be neither formed nor destroyed in the body. Of the various chemicals used for this determination (thiocyanate, sucrose, inulin, bromide, iodide, sulfate and the radioisotopes), none is completely satisfactory. Sucrose and inulin are rapidly excreted; thiocyanate, bromide, iodide and radiochloride enter digestive juices and all chloride-containing cells, e.g., red and white blood cells, and radiosodium eventually enters all sodium-containing cells and the solid phase of bone.

Since sodium and chloride are known to be predominately extracellular ions, their radioisotopes should theoretically constitute the best tools for estimation of extracellular water. It has been implied above and seems, indeed, to be the case that neither sodium nor chloride is entirely confined to the extracellular phases of all tissues of the body. Both ions may reside inside some cells (e.g., leukocytes, dog red cells and glandular tissues), while in other cell types either chloride (connective tissue, pyloric mucosa and testes) or sodium (intestinal wall, etc.) may be intracellularly located. With the exception of skin, the volume of all these tissues is small compared with that of muscle which, because of its bulk, must be the greatest single factor influencing the electrolyte pattern of the body as a whole. There is, to be sure, some excess sodium in muscle, but not only is this quantity variable but it is not large and its location is not yet satisfactorily settled. In general, the radioisotopes exchange slowly with the so-called intracellular ions. Hence, without a detailed analysis of the complex structure of each tissue (see (18) and (19)), it will suffice to say that the behaviour of injected radiosodium and radiochloride demonstrates the fact that they are predominately extracellular ions.

Some observations may be mentioned which illustrate the behaviour of the isotopes of these elements. Both radioactive sodium and chloride cross capillary walls (7, 13, 22) and enter the extracellular spaces of most tissues in a few minutes (18, 19), but enter brain tissue, cerebrospinal fluid and eye humours more slowly (12, 18, 19, 27, 29). Although radioactive chloride penetrates red cells rapidly (7, 25), it exchanges more slowly with the intracellular chloride which testes and pyloric mucosa seem to possess (19). On the other hand, red cells which contain intracellular sodium permit a relatively slow penetration of radiosodium (6, 18). Both isotopes enter digestive juices (3, 23, 28), but the interference with their usefulness for the determination of extracellular fluid which would, for this reason, result is minimized by assuring that the subject is in a postabsorptive condition. Actually, radiosodium is not excreted into saliva and gastric juice to nearly the same extent as thiocyanate, although both enter serous effusions (16).

In addition to the foregoing observations, which illustrate the extracellular nature of sodium and chloride, there is ample evidence that after injection the radioisotopes of both substances leave the plasma in an orderly fashion until an approximately constant value is reached. This occurs in about 10 min in small animals such as the guinea pig (Fig. 1, *C*) (7, 22), in 40–60 min in dogs (*D*) (11, 12, 29, 31) and in $2^1/_2$ to 3 hr in man (*A* and *B*) (9, 17). In practice, 60–90 min is usually allowed to insure complete equilibrium in laboratory animals. Experiments are likewise available which show that negligible amounts of radiosodium are excreted in the periods of time required for measurement of extracellular fluid volume (4, 5, 9, 26, 31).

Hence radiosodium and chloride fulfil all of the requirements very well. The slow accumulation of radiosodium in bone presents the only objection to its usefulness for measurement of extracellular fluid, but this is not serious in short experiments. That half of the total body sodium is in the skeleton and that part of this bone sodium is fixed in the insoluble mineral portion, perhaps as a part of the apatite lattice, has been known for some years. The lability of this bone sodium, i.e., the ease with which it will exchange with plasma sodium, was impressively demonstrated by the uptake of radiosodium by bone (11, 15, 16, 18). Obviously, if any appreciable amount of the plasma radiosodium enters bone during the experimental period a correction should be made. Unfortunately it cannot be decided whether or not such a correction is generally essential because accurate studies of the rate of entry of radiosodium into bone are not available. Kaltreider *et al.* (16) estimated that in 12 hr after injection into human beings, about 20% of the total isotope in the body was in the insoluble phase of bone. They believe that a correction for this amount was necessary in estimations of extracellular fluid volume. Undoubtedly the accumulation in bone is largely responsible for the slow decline in the plasma concentration of radiosodium observed after its initial rapid exit from the blood into the extravascular spaces. However, in view of the

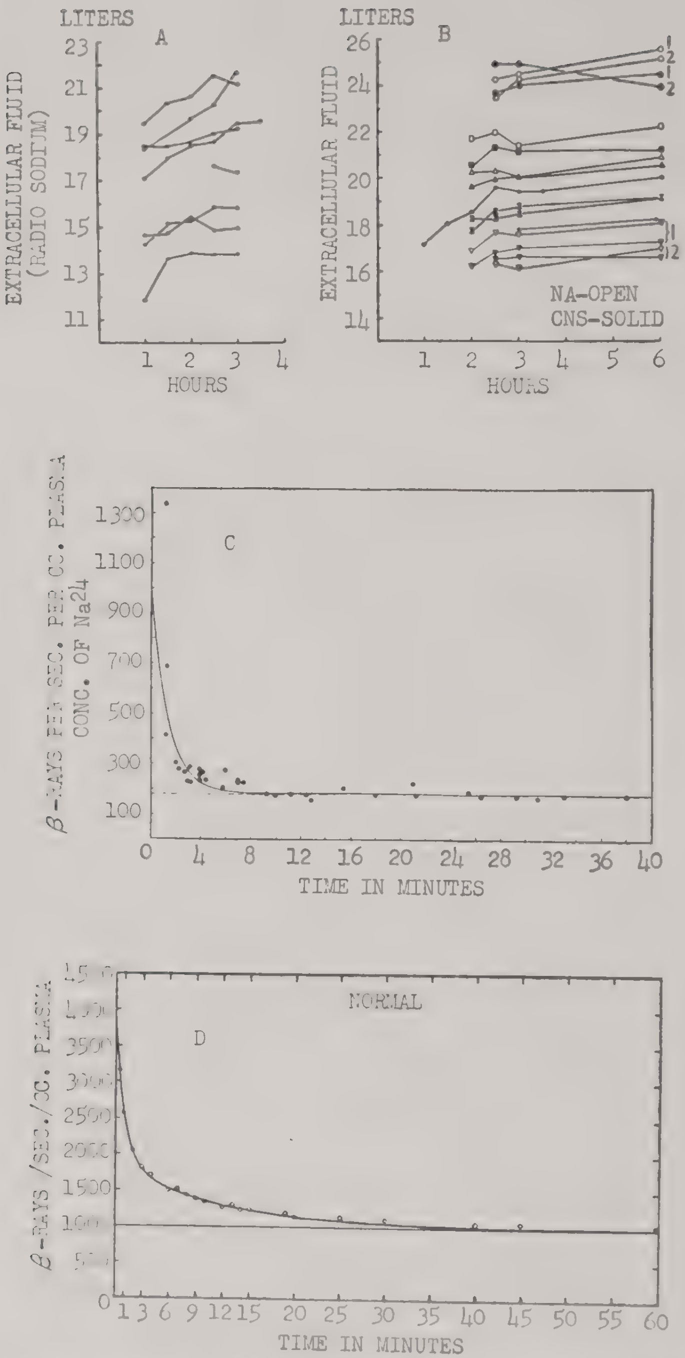

FIG. 1.—*A* and *B*, change with time in extracellular fluid volumes in normal human subjects as measured by (*A*) Na^{24} and (*B*) Na^{24} and thiocyanate (after Kaltreider *et al.* (16)). Disappearance of intravenously injected Na^{24} from plasma of guinea-pigs (after Merrell *et al.* (22)) and dogs (after Gellhorn *et al.* (11)). Note that equilibrium is reached in 10 min in the guinea-pig, 40 min in the dog and 2½–3 hr in man.

large error in the estimation, lack of precise information regarding the weight of the skeleton and the fact that entrance into bone is a delayed phenomenon, we believe that a correction for bone sodium is unwarranted at this time. It is conceivable that the lability of this bone sodium could be responsible for a more serious error if the subject were not in sodium balance (4, 26). The most that can be said is that since no experiments have been performed which allow an evaluation of such an error, the possibility of its significance must be borne in mind.

Radiosodium and radiochloride have 2 additional advantages for the determination of extracellular fluid volume. (1) They are rapidly and easily measured. (2) Since sodium and chloride are normal constituents of all tissues and fluids, they can be injected in a small amount of isotonic saline and there is little danger of toxic effects even in cases of kidney impairment, or when repeated trials are necessary.

The isotope of choice.—Several isotopes of sodium and chloride are available. Of these, Na^{24} is at present the preferable isotope. Those theoretically available are Na^{22}, half-life 3 yr (cyclotron); Na^{24}, half-life 14.8 hr (cyclotron and uranium pile); Cl^{34}, half-life 33 min; Cl^{36}, half-life 2×10^6 yr (uranium pile) and Cl^{38}, half-life 37 min (cyclotron). Of these, only Na^{24}, Na^{22} and Cl^{38} have been used in biologic experiments. More detailed information about these isotopes may be obtained from several sources (17, 24).

The long half-life of Na^{22} and the predilection of sodium for bone where blood-forming tissue is located makes its use more hazardous than that of Na^{24}, particularly if repeated doses seem indicated. Na^{22} is not immediately eliminated, as shown by the report that half the dose given was excreted in 9–42 days, average 28.8 days (26). The short half-life of Cl^{38}, on the other hand, reduces its usefulness because each count for each sample must be corrected for radioactive decay and because it is impossible to use this isotope except in laboratories in close proximity to the production source.

The various isotopes are prepared in a variety of ways. Na^{22} results from deuteron bombardment of magnesium metal in the cyclotron. Na^{24} has been prepared in the cyclotron using as a target for deuterons, metallic sodium, NaCl or NaOH. When NaCl is used Cl^{38} is formed in addition to Na^{24} and the experiment cannot be performed until the chloride radioactivity has dissipated. NaCl or Na_2CO_3 can be placed in the uranium pile for the preparation of Na^{24}. It should be observed that chloride has a high capture cross-section and, in the pile, seriously competes with sodium for available neutrons. In addition, nuclear reactions resulting in the formation of S^{35} and P^{32} are possible. In our experience S^{35} was a serious contaminant when NaCl was placed in the pile (30).

The samples of Cl^{38} which have been most frequently used were produced by bombarding lithium chloride with deuterons. Without further treatment a solution of $LiCl^{38}$ was injected. Lithium too becomes radioactive, but since the half-life of the isotope is only 0.88 sec its radioactivity had disappeared before the experiments commenced. KCl and $CaCl_2$ have likewise been used as targets for deuterons in the formation of Cl^{38}. These require chemical processing before use (see p. 62), which is a disadvantage when the half-life is so short. Cl^{36} has not been produced in any quantity.

DOSE TO BE ADMINISTERED

Two factors must be considered in estimating the dose to be administered. (1) A sufficient amount must be given to permit convenient counting of the samples at the conclusion of the experiment. (2) This amount must be small enough to insure safety to the subject from overirradiation.

Counts necessary for the experiment.—A sample calculation may serve to illustrate the number of counts necessary for accurate determination of extracellular fluid volume. The lowest permissible counting rate is about 3 times the background count, while the optimal count, for convenience and accuracy when the Geiger-Müller counter is used, may be closer to 20 times the background. In our laboratory the background counts vary from 18 to 28/min when taken with a commercial self-quenching G.-M. tube with a thin mica window, the tube being enclosed in a lead shield. Sufficient counts must then be injected so that, after their distribution throughout all of the extracellular water (about 25% of the body weight), not less than 500 counts/min will be in 1 ml of plasma at the time of counting.

The volume of extracellular water in a 70 kg man is about 17,500 g or ml. Hence the total counts in the body at the time of counting should be at least $500 \times 17{,}500 = 8.75 \times 10^6$. If the half-life of the isotope is short compared with the duration of the experiment, this fact too must be considered. Suppose that 4 hr elapse between the time of administration of Cl^{38} (half-life 37 min) and the period of counting; in these circumstances the sample will decay to half its value about 6 times. Hence the total number of Cl^{38} counts injected should be

$$Cl^{38}\text{ counts/min} = 8.75 \times 10^6 \times 2^6 = 5.6 \times 10^8$$

In the case of Na^{24} (half-life 14.8 hr), the radioactivity will decay to 83% of its value in 4 hr or to half its value in 14.8 hr. For an experiment of 4 hr duration the number of counts of Na^{24} injected should be

$$Na^{24} \text{ counts/min} = 8.76 \times 10^6 \times \frac{100}{83} = 1.05 \times 10^7$$

If the counting cannot be carried out 4 hr after the injection it is advisable to increase the dose, even as much as 10-fold, which, as we shall see later, is still within the limits of safety for a single dose. If the plasma count is too high at the time of counting it can always be appropriately diluted.

Calculation of safe dose.—Before the radiation dose to which the subject is being exposed can be calculated, it is necessary to know the number of disintegrations/sec which have been injected. This number will be considerably higher than the number of counts because the counting apparatus is not 100% efficient. Its efficiency can be determined by counting a weighed standard of known activity; e.g., 3.5 g of U_3O_8 supplies 3.7×10^4 disintegrations/sec (see ref. 17, p. 91). If the apparatus were 40% efficient the number of disintegrations injected into a 70 kg man would have been 2.5 times the estimated counts.

$$\begin{aligned} 1 \text{ millicurie (mc)} &= 3.70 \times 10^7 \text{ disintegrations/sec} \\ &= 2.22 \times 10^9 \text{ disintegrations/min} \\ 1 \text{ microcurie } (\mu c) &= 3.7 \times 10^4 \text{ disintegrations/sec} \\ &= 2.22 \times 10^6 \text{ disintegrations/min} \end{aligned}$$

Therefore

$$Cl^{38} \text{ injected } (\mu c) = \frac{5.6 \times 10^8 \times 2.5}{2.22 \times 10^6} = 6.3 \times 10^2\ \mu c$$

$$Na^{24} \text{ injected } (\mu c) = \frac{1.05 \times 10^7 \times 2.5}{2.22 \times 10^6} = 11.8\ \mu c$$

These amounts represent the doses to be given a 70 kg man to provide adequate counts per ml of plasma (using a 40% efficient counter) in 4 hr after the injection. The investigator must assure himself that the amounts do not exceed the accepted limits of safety for radiation dosage. The older value of a "safe dose" of 0.1 r/day has been reduced to 0.3 r/week. (For recent discussions of dosimetry, see 1, 2, 20, 21, 24.)

A roentgen is the quantity of X- or gamma radiation such that the associated corpuscular emission in 1 cc of air (0.001293 g) at N.T.P. produces ions (2.08×10^9 ion pairs) carrying 1 electrostatic unit (esu) of charge. In 1 g of air the roentgen produces $2.08 \times 10^9 \times 774 = 1.61 \times 10^{12}$ ion pairs. Since roentgen is a term which applies only to X- and gamma radiation and only to air as the absorbing medium, another term, the equivalent roentgen or the roentgen equivalent physical (rep) has been introduced. This applies to X- and gamma radiation, alpha, beta, proton or neutron radiation and is that amount of ionizing radiation which will give rise to 1.61×10^{12} ion pairs in media other than air, i.e., in 1 cc of water or 1 cc of tissue of unit density.

The energy absorbed per ion pair is 32 electron volts (ev). Hence a dose of 1 r received at any point means that $1.61 \times 10^{12} \times 32 = 5.18 \times 10^{13}$ ev or 5.18×10^{7} Mev or 83 ergs of energy is absorbed per g of air and a dose of 1 rep results in the absorption of the same amounts of energy per g of tissue. The difference between the units r and rep is made clear when it is appreciated that a dose of 1 r of gamma rays causes the absorption of 83 ergs/g of air, 93 ergs/g of muscle and several hundred ergs/g of bone. Despite the variation in energy absorbed in different substances the dose is still 1 r if it produces 1 esu of charge per cc of air at the point considered. The roentgen equivalent physical, on the other hand, is always the dose which causes an absorption of 83 ergs/g of tissue and is independent of the tissue composition. In muscle 1 r of radiation corresponds to 1.1 rep.

To determine the safety of the dose necessary for the experiment, a calculation must be made of the total amount of energy liberated per g of tissue from the time of injection until all of the isotope has disappeared from the body by radioactive decay or elimination.

The energy (E_d) made available per g of tissue per sec by a beta emitter is given by the product of the number of disintegrations per sec per g of tissue and the average energy ($\bar{E}_\beta$) of each disintegration. The maximum energy ($E_{\max}$) in Mev is given in any table listing radioactive isotopes, and $\bar{E}_\beta = 1/3$ or $2/5$ $E_{\max}$, i.e., $0.4 \times E_{\max}$. Therefore

$$E_d = 3.7 \times 10^4 \times N \times 0.4\, E_{\max} \text{ in Mev/sec/g}$$

where $N = \mu c/g$. If the isotope injected for the determination of extracellular volume is not eliminated from the body until it has disappeared due to radioactive decay, as would be the case with Cl^{38}, it is necessary to calculate the radiation dose during complete decay. The total number of beta particles emitted during decay to zero activity is the number of betas/sec multiplied by the average life of the atom in question, measured in seconds.

$$\text{av. life} = \frac{\text{half-life (sec)}}{0.693}$$

Therefore the energy from the total radiation dose,

$$E_d = 3.7 \times 10^4 \times 0.4\, E_{\max} \times N \times \frac{\text{half-life (sec)}}{0.693} \text{ Mev/g}$$

Since a dose of 1 rep results in the absorption of 5.18×10^7 Mev/g, this total dose, D, may be expressed in rep units.

$$D = \frac{3.7 \times 10^4}{5.18 \times 10^7} \times 0.4 \times E_{\max} \times N \times \frac{\text{half-life (sec)}}{0.693} \text{ rep}$$

Finally the distribution of these isotopes throughout the body must be considered. Since the radioactive atoms enter the extracellular spaces everywhere, their beta particles will be almost uniformly distributed. Hence the dose injected will be spread throughout the entire mass of the individual, and if the half-life of the isotope is stated in hours, the total dose is

$$\begin{aligned} D &= \frac{3.7 \times 10^4}{5.18 \times 10^7} \times \frac{0.4}{0.693} \times 60 \times 60 \times N \times E_{\max} \times \frac{\text{half-life (hr)}}{\text{body wt. (g)}} \\ &= 1.48 \times N \times E_{\max} \times \frac{\text{half-life (hr)}}{\text{body wt. (g)}} \text{ rep} \end{aligned}$$

If the half-life is given in days, the dose is

$$D = 35.4 \times N \times E_{\max} \times \frac{\text{half-life (days)}}{\text{body wt. (g)}} \text{ rep}$$

The dose of Na^{24} administered to a 70 kg man may be calculated as follows when $N = 11.8\ \mu c$, $E_{\max} = 1.4$ Mev, half-life $= 14.8$ hr,

$$\begin{aligned} D &= 1.48 \times 11.8 \times 1.4 \times \frac{14.8}{70{,}000} \text{ rep} \\ &= 0.0052 \text{ rep} \end{aligned}$$

Similarly, the total dose of Cl^{38} is calculated as follows when $N = 6.3 \times 10^2\ \mu c$, $E_{\max} = 3.5$ (av.) Mev, half-life $= 0.617$ hr,

$$\begin{aligned} D &= 1.48 \times 6.3 \times 10^2 \times 3.5 \times \frac{0.617}{70{,}000} \text{ rep} \\ &= 0.0287 \text{ rep} \end{aligned}$$

Obviously, accepting uniform distribution throughout the body, the total radiation dose from the beta particles of each isotope is well within the safety limits of 0.3 r/week. Even if some Na^{24} did accumulate in bone it could do so several-fold before dangerous dosages would be experienced.

This calculation deals with beta particles only. A similar estimation of the energies due to gamma rays can be made (21, 24), but for this paper it will perhaps suffice to refer the reader to a calculation by Marinelli (21) in which he considers both beta and gamma radiations of Na^{24}. He estimates that for a tolerance dose of 0.1 r/day only 0.0013 μc/g should be given, or 91 μc/70 kg, which is about 8 times as great as that necessary for a Na^{24} experiment. No account is taken of the small amount of the isotopes which will be eliminated during the experiments (5, 9, 31). A complete discussion of the elimination of Na^{22} has been published by Burch and his colleagues (4, 26) in 2 papers dealing with "biologic" decay of isotopes.

Finally, in a discussion of safe doses of radiation attention should be drawn to the fact that use of amounts as large as 1–10 rep is justified if not repeated too frequently, especially in the case of an isotope with a short half-life such as Na^{24} and Cl^{38}.

PROCEDURE

a) Preparation of sample for injection.—In most instances the salt used as a target for production of a radioisotope must be processed chemically before it can be injected into animals. A solution of $Na^{24}Cl$ can, of course, be used as soon as the Cl^{38} activity has disappeared. Metallic sodium can be washed off the target holder with 50% alcohol in an atmosphere of CO_2, the washings acidified with HCl and evaporated to dryness to remove excess HCl. Any radioactive heavy metals contaminating the resulting NaCl may be precipitated in a variety of ways, e.g., with H_2S. It is more common to use NaOH as target material in the cyclotron or Na_2CO_3 in the uranium pile. Both of these salts must be changed to NaCl by the addition of HCl; the excess HCl is then driven off by evaporation to dryness. The entire operation can be carried out in a small tared beaker and the final weight of the dry salt estimated. A measured amount of distilled water is added to the beaker to make an approximately isotonic solution of NaCl, i.e., 0.15M or 0.85–1%. This is the stock solution subsequently used for injection.

The radioisotope Cl^{38}, prepared by bombarding lithium chloride with deuterons, has frequently been used. It is dissolved in water to make 0.15M solution and injected as soon as the lithium radioactivity (half-life 0.88 sec) has dissipated. KCl (7) and $CaCl_2$ (29) have been used as targets. The $CaCl_2$ is treated with H_2SO_4 and the HCl^{38}, after being distilled off, is neutralized with NaOH. When KCl is used the chloride is precipitated as AgCl and changed to $ZnCl_2$ after reduction of the silver; the zinc is then precipitated as $ZnCO_3$ by the addition of Na_2CO_3, and the $NaCl^{38}$ solution is decanted off (for further detail see reference (7)). Both of these procedures are time-consuming, which is a matter of some importance when the isotope is short-lived.

It is advisable to observe certain simple safety precautions during the preparation of these strongly radioactive samples. The investigator should wear rubber gloves, handle the sample with tongs and keep it shielded by lead bricks as much of the time as possible.

He should work on a laboratory bench covered with easily removable heavy paper, should use rubber bulbs on pipets and should maintain a special receptacle for contaminated radioactive glassware.

b) *Collection and preparation of samples for counting.*—A rough estimation of the activity of the stock solution must be obtained in order to dilute it appropriately. The diluted solution is then filtered through a bacterial filter, such as a low porosity sintered glass filter funnel, just before injection. The amount of solution which is injected should be carefully measured, using a good syringe which has previously been accurately calibrated for delivery.

With sterile precautions the radioactive solution is injected intravenously into a subject in a postabsorptive condition. The site of injection is a matter of choice. It is advisable, however, not to use the same site for removing blood for analysis because of the danger of contamination. Although a subject in normal or low sodium balance will not excrete a significant amount of the isotope, nevertheless for most accurate estimations the experiment should begin with the bladder empty, and the urine should be collected during the experimental period.

Blood samples should be procured from human subjects 1, 2 or 3 hr after the injection to be assured that equilibrium has been established. In oedematous patients, 6–9 hr seems necessary for the radioisotopes to spread through the entire extracellular water. A shorter period of 1–1½ hr is adequate for most animals. Some suitable dry anticoagulant is used such as heparin (0.2 mg/ml of blood) or the oxalate mixture of Heller and Paul* (14). Neither of these anticoagulants disturbs the relationship between cells and plasma. All blood samples should be centrifuged under paraffin oil for 15 min and the plasma removed.

A variety of procedures for preparing the samples for counting are in current use. If an immersion type of counter is used, samples of plasma or urine containing either isotope may be suitably diluted and counted directly. The protein content of plasma is too small to warrant the application of any ashing procedures. Similarly, when a mica window G.-M. counting tube is used, the original or diluted plasma, urine or Na^{24} solution may be counted directly. Some investigators measure an appropriate amount of the solution (0.1–1.0 ml) into a shallow aluminum dish and dry these samples under an infra-red lamp. When dry they are placed in a holder close to the mica window for counting. Others drop a measured volume of fluid on filter paper discs which are placed on metal discs. These can be counted wet if evaporation is avoided or can be dried completely. If the dry filter paper discs tend to curl, they are placed in the centre of the top of a small, solid metal cylinder and covered with a sheet of thin plastic film; the plastic is held tightly over the edge of the cylinder by a metal ring which fits the outside of the cylinder snugly.

---

* Made by dissolving 3 g of ammonium oxalate and 2 g of potassium oxalate in 250 ml of water (0.25 ml/5 ml of blood).

c) *Counting of samples and calculation of results.*—The number and variety of methods of counting radioactive samples precludes a detailed description of them here. Because the mica window G.-M. tube is in common use, the procedure pertaining to it will be outlined. In addition to counting the biologic samples obtained from the subject, it is necessary to obtain a background count, to check the sensitivity of the G.-M. tube by counting a uranium standard and to count a sample of the radioactive solution injected frequently enough to establish its identity by plotting a decay curve.

TABLE 1.—DATA NECESSARY FOR DETERMINATION OF EXTRACELLULAR WATER BY Na^{24}

Subject:
Weight:
Vol. of solution injected:
Vol. of urine collected:

(1) TIME OF COUNTING	(2) SAMPLE	(3) DILUTION FACTOR	(4) TIME FOR 3072 CT (MIN)	(5) CT/MIN	(6) NET CT/MIN*	(7) DECAY CORRECTION	(8) NET CT/MIN/ML AT 0 TIME
	Background						
	Uranium						
	Na^{24} sol.						
	Plasma						
	Urine						
	Na^{24} sol.						
	Urine						
	Plasma						
	Na^{24} sol.						
	Uranium						
	Background						

* Net counts/min = sample counts in column (5) minus background count.

The data necessary for these experiments are demonstrated in Table 1. If only a few Na^{24} samples of approximately the same activity are to be measured, they may be counted in the order listed in the table. When the duplicate counts are averaged they are automatically corrected for decay.

If corrections for radioactive decay are necessary, a decay curve should be plotted on semilogarithmic paper with the counting rate (in % of that at zero time) on the logarithmic Y-axis and time on the X-axis. The data plotted are derived from the formula $N_t = N_0{}^{-\lambda t}$, where N_0 and N_t are the number of disintegrating atoms present at zero time and time t, respectively, and λ is the decay constant for the isotope. The equation of the straight line obtained is $\log \frac{N_t}{N_0} = \frac{-\lambda}{2.303} \cdot t$, and $\frac{\lambda}{2.303}$ is the slope of the line. The value of $\lambda = 0.693 \div$ half-life. The graph is simply constructed by drawing a straight line between 2 points, A (100%, 0) and B (50%, 14.8 hr) for Na^{24} and A (100%, 0) and C (50%, 37 min) for Cl^{38}. Zero time can be taken as the time of injection or the time at which the first sample is counted.

When samples contain Cl^{38}, the use of a decay curve is essential. For the time at which the sample was counted it is advisable to use the mean point of the interval required for counting.

The total counts injected and excreted are estimated and, using the following formula, the volume of extracellular water, $(H_2O)_E$, is calculated:

$$(H_2O)_E Na^{24} \text{ or } (H_2O)_E Cl^{38} \text{ (g/100 g body wt.)}$$

$$= \frac{\text{counts injected} - \text{counts excreted}}{\text{plasma counts/ml of plasma}} \times \frac{100}{\text{body wt. (g)}} \times 0.93$$

where 0.93 is a correction for the water content of the plasma. This is sufficiently accurate without a determination of the water content of plasma unless a widely divergent value is expected. The Gibbs-Donnan distribution ratio of 0.95, by which the equation is multiplied in the case of Cl^{38} and divided in the case of Na^{24}, can be neglected since it is a small correction compared with the error of the procedure.

Values obtained and critique of method.—Since radioactive decay is a random process, the accuracy of the counting procedure itself is limited by statistical fluctuations. The fluctuations can be related to a fundamental parameter of the data, the standard deviation σ, which is equal to $\sqrt{N}$, where N = number of counts. If $N = 1024$, the value of σ is 3.2%, which means that approximately $^2/_3$ of the activity measurements are within 2% of the genuine activity. The probable error, i.e., $0.67\sqrt{N}$, is also frequently used to describe the counting error.

It is unfortunate that too few determinations of extracellular water have been made to allow a similar concise statement of the accuracy of the entire procedure. Table 2 gives average values and range of these values found by several investigators for a variety of species. Only the data obtained by intravenous injection of the isotope have been recorded here, since methods using intraperitoneal injection are subject to greater error. The variation is great, which is not unexpected because the extracellular fluid volume expressed as per cent of the body weight varies with the degree of obesity of the individual. Less variability is found when the results are expressed per unit of surface area. Repeated volumes on the same individual, however, agree very well even when expressed per unit of body weight. Study of 6 individuals showed that the difference between duplicate determinations should not exceed ±0.98 l or ±4.9% when the average volume was 19.9 l (16). Age also is an important factor contributing to the variability of extracellular fluid measurements. It is now well established that young individuals (9) have a much higher extracellular space than adults of the same species (Table 2).

TABLE 2.—VALUES OF EXTRACELLULAR FLUID VOLUMES, $(H_2O)_E$, DETERMINED BY USE OF CL^{38}, NA^{24} AND NA^{22}*

(1) SPECIES	(2) NO. OF SUBJECTS	(3) $(H_2O)_E$ IN % BODY WT.	
		Av.	Range
Cl^{38}			
Rabbit (19)	4	21	17–26
Rabbit (13)	2	27	22–33
Dog (31)	3	25	
Na^{24}			
Rabbit (13, 19)	2	28	25–31
Dog (31)	3	28 [36]	
Dog (12)		25	22–29
Man (16)	14	25 [25]	22–30
Man (9)	5	27 [23]	25–28
Infant (9)	3	43 [41]	31–52
Na^{22}			
Rat (8)	24	27	±0.7†
Infant (10)	3	44	41–46

* Parenthetical figures in column (1) refer to a bibliographic reference. Those bracketed in column (3) are the average values of $(H_2O)_E$ measured by the thiocyanate method, for the same individuals who received Na^{24}.

† Standard error of the mean.

Three groups whose figures are given in Table 2 determined the volume of extracellular fluid by thiocyanate on the same individuals as those receiving Na^{24}. The approximate similarity of the 2 values is evident from the figures listed in column (3).

In conclusion it may be stated that, although like other procedures the radioisotope method does not give a precise estimate of the extracellular fluid, it affords at least as reliable values as any of the other procedures. Radioisotopes satisfy all of the essential criteria and in addition can be rapidly and accurately measured.

NOTE.—This section was reviewed by W. O. Fenn.

REFERENCES

1. Bale, W. F.: Health Hazards in the Use of Radioactive Isotopes, in *A Symposium on the Use of Isotopes in Biology and Medicine* (Madison, Wis.: University of Wisconsin Press, 1948), p. 395.
2. Brues, A. M.: Biological hazards and toxicity of radioactive isotopes, J. Clin. Investigation 28: 1286, 1949.
3. Brunschwig, A., and Schmitz, R. L.: Rapidity of passage of chloride ion from blood into gastric juice of stimulated stomach, Proc. Soc. Exper. Biol. & Med. 43: 438, 1940.
4. Burch, G. E.; Threefoot, S. A., and Cronvich, J. A.: Theoretic considerations of biologic decay rates of isotopes, J. Lab. & Clin. Med. 34: 14, 1949.
5. Burch, G. E.; Threefoot, S. A., and Reaser, P. B.: Some aspects of renal excretion of Na^{24} by normal subjects and by patients with congestive heart failure, Stanford M. Bull. 6: 81, 1948.
6. Cohn, W. E., and Cohn, E. T.: Permeability of red corpuscles of the dog to sodium ion, Proc. Soc. Exper. Biol. & Med. 41: 445, 1939.
7. Cowie, D. B.; Flexner, L. B., and Wilde, W. S.: Capillary permeability: Rate of transcapillary exchange of chloride in the guinea pig as determined with radiochloride, Am. J. Physiol. 158: 231, 1949.
8. Cuthbertson, E. M., and Greenberg, D. M.: Chemical and pathological changes in dietary chloride deficiency in the rat, J. Biol. Chem. 160: 83, 1945.
9. Fellers, F. X., *et al.:* Changes in the thiocyanate and $sodium^{24}$ spaces during growth, Pediatrics 3: 622, 1949.
10. Flexner, L. B., *et al.:* The estimation of total and extracellular body water in the newborn human infant and radioactive sodium and deuterium oxide, J. Pediat. 30: 413, 1947.
11. Gellhorn, A.; Merrell, M., and Rankin, R. M.: The rate of transcapillary exchange of sodium in normal and shocked dogs, Am. J. Physiol. 142: 407, 1944.
12. Greenberg, D. M., *et al.:* A study with radioactive isotopes of the permeability of the blood cerebrospinal fluid barrier to ions, Am. J. Physiol. 140: 47, 1943.
13. Hahn, L., and Hevesy, G.: Rate of penetration of ions through the capillary wall, Acta physiol. Scandinav. 1: 347, 1941.
14. Heller, V. G., and Paul, H.: Changes in cell volume produced by varying concentrations of different anticoagulants, J. Lab. & Clin. Med. 19: 777, 1934.
15. Hodge, H. C., *et al.:* The nature of the insoluble sodium of bone: The adsorption of sodium at forty degrees by bone, dentin, enamel, and hydroxyapatite as shown by the radioactive isotope, J. Biol. Chem. 148: 321, 1943.
16. Kaltreider, N. L., *et al.:* Determination of the volume of the extracellular fluid of the body with radioactive sodium, J. Exper. Med. 74: 569, 1941.
17. Kamen, M. D.: *Radioactive Tracers in Biology* (New York: Academic Press, 1947).
18. Manery, J. F., and Bale, W. F.: The penetration of radioactive sodium and phosphorus into the extra- and intra-cellular phases of tissues, Am. J. Physiol. 132: 215, 1941.

19. Manery, J. F., and Haege, L. F.: The extent to which radioactive chloride penetrates tissues, and its significance, Am. J. Physiol. 134: 83, 1941.
20. Marinelli, L. D.: Dosage determinations with radioactive isotopes, Am. J. Roentgenol. 47: 210, 1942.
21. Marinelli, L. D.: Dosage determination in the use of radioactive isotopes, J. Clin. Investigation 28: 1271, 1949.
22. Merrell, M.; Gellhorn, A., and Flexner, L. B.: Studies on rates of exchange of substances between the blood and intravascular fluid: 11. The exchange of sodium in the guinea pig, J. Biol. Chem. 153: 83, 1944.
23. Montgomery, M. L.; Sheline, G. E., and Chaikoff, I. L.: Elimination of sodium in pancreatic juice as measured by radioactive sodium, Am. J. Physiol. 131: 578, 1941.
24. Siri, W. E.: *Isotopic Tracers and Nuclear Radiations with Applications to Biology and Medicine* (New York: McGraw-Hill Book Company, Inc., 1949).
25. Smith, P. K.; Eisenman, A. J., and Winkler, A. W.: Permeability of human erythrocytes to radioactive chloride and to bromide and iodide, J. Biol. Chem. 141: 555, 1941.
26. Threefoot, S. A.; Burch, G. E., and Reaser, P. B.: The biologic decay periods of sodium in normal man, in patients with congestive heart failure, and in patients with nephrotic syndrome as determined by Na^{22} as the tracer, J. Lab. & Clin. Med. 34: 1, 1949.
27. Visscher, M. B., and Carr, C.: The rate of entrance of radio sodium into the aqueous humor and cerebrospinal fluid, Am. J. Physiol. 142: 27, 1944.
28. Visscher, M. B., *et al.:* Isotopic tracer studies on the movement of water and ions between intestinal lumen and blood, Am. J. Physiol. 142: 550, 1944.
29. Wang, J. C.: Penetration of radioactive sodium and chloride into cerebrospinal fluid and aqueous humor, J. Gen. Physiol. 31: 259, 1948.
30. Wilson, D. L., and Manery, J. F.: The permeability of rabbit leucocytes to sodium, potassium and chloride, J. Cell. & Comp. Physiol. 34: 493, 1949.
31. Winkler, A. W.; Elkinton, J. R., and Eisenman, A. J.: Comparison of sulfocyanate with radioactive chloride and sodium in the measurement of extracellular fluid, Am. J. Physiol. 139: 239, 1943.

Biologic Method for Estimation of Calcium Ion Concentration*

FRANKLIN C. McLEAN, *The University of Chicago*

There is as yet no satisfactory physical or chemical method for determining the concentration of calcium ions in solution, except under very limited conditions. Joseph (8) described an amalgam electrode of the third kind which gives satisfactory results in certain solutions, but not in biologic fluids containing bicarbonate ions (9). McLean and Hastings (12) described in 1934 a biologic method for estimation of calcium ion concentration, and the present description is taken largely from that paper. Although this

---

* This work was aided by a grant from the Josiah Macy, Jr., Foundation.

method is subject to some limitations, to be noted, it has proved useful in a number of chemical, physiologic and clinical problems (7, 11, 13, 15) and has still to be replaced by a more satisfactory direct chemical method. The method uses the isolated heart of the frog as a biologic indicator and is applicable to any solution or biologic fluid capable of sustaining the action of the frog's heart provided the concentration of calcium ions is within the range of sensitivity of the heart.

The sensitivity of the frog's heart to changes in the calcium content of its nutrient fluid has long been known. Straub (16) commented that this sensitivity could be made use of to titrate calcium concentrations. Trendelenburg and Goebel (17) used it to demonstrate the reduction in calcium in the serum of the cat in parathyroid tetany. Klinke (10), in his comprehensive review, mentioned the frog's heart as a possible biologic indicator of calcium ion concentrations, but expressed doubt, based partly on the work of Günther and Huebner (6), that its contraction is affected by calcium in the ionized form alone. The use made of the frog's heart by Clark, Percival and Stewart (2) in a study of calcium and citrate mixtures is referred to in detail elsewhere (7).

The method here described is the result of an attempt to utilize this sensitivity of the frog's heart, in a strictly quantitative manner, for estimation of calcium ion concentrations in certain saline solutions and biologic fluids. Its validity is based on the following assumptions or demonstrations:

1. That all calcium in the solutions used as standards of reference is present in ionized form, even when these solutions are supersaturated with respect to $CaCO_3$. Since all Ca^{++} observations are expressed in terms of the standard solutions, any error due to this assumption would have a corresponding effect on the Ca^{++} concentrations estimated by the method.

2. That the frog's heart is sensitive to changes in concentration of Ca^{++} but not to changes in concentration of calcium in non-ionized form. That this is true when calcium is combined with citrate is shown elsewhere (7). That it is true when calcium is combined with protein has also been shown (14).

3. That, other conditions being identical, 2 solutions inducing an identical response from the frog's heart, in a range of Ca^{++} concentration in which the heart is able to respond to changes in either direction, have an identical concentration of Ca^{++}.

4. That in the case of biologic fluids, it being impossible to make the solutions of reference exactly comparable, the results may be regarded as valid provided certain precautions, to be described, are observed.

The method consists essentially in the direct comparison and matching of known solutions with solutions or biologic fluids of unknown Ca^{++} concentration, the criterion for equal Ca^{++} concentration being equal amplitude of contraction of the ventricle of the isolated heart of the frog, as recorded on a drum. It is emphasized that the method depends on direct matching and does not involve calculations from 1 set of conditions to another. Provided, therefore, the heart used is sensitive to changes within the range in which Ca^{++} is present, a condition which must in each instance be demonstrated, the method is independent of the particular state of activity, or particular amplitude of contraction, of the particular heart used as an indicator.

PROCEDURE

a) *Preparation.*—The preparation is the isolated heart of the frog, suspended from a modified Straub cannula and so arranged that the amplitude of contraction of the ventricle will be recorded, greatly amplified, on a moving drum. Healthy, full-blooded frogs of medium size are chosen. Frogs showing edema of the legs are particularly to be avoided.

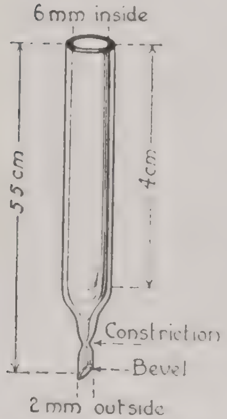

FIG. 1.—Modified Straub cannula.

A frog is pithed and the heart exposed. A straight cannula (Fig. 1) 5.0–5.5 cm long overall, with internal diameter 6.0 mm, capacity about 1.0 cc and a tip with outside diameter not to exceed 2 mm., is inserted through the left branch of the aorta into the ventricle, where it is held by a ligature around the aorta. The heart is raised from the body, a 2d ligature is passed around all the tissues to which the heart is attached, with care not to injure the auricles, and the attachments are severed beyond the ligature. The cannula is filled with a suitable fluid, and the fluid repeatedly changed until all blood has been washed out. For changing the fluid, a medicine dropper with a tip long enough to reach the constricted portion of the cannula (see detail in Fig. 1) is employed.

If the preparation has been properly made, the fluid in the cannula will move with an excursion of several millimeters with each beat of the heart, and there will be no escape of fluid. If fluid escapes, indicating damage to the preparation, a new heart is usually required. The chief source of difficulty in making the preparations is in reaching the cavity of the ventricle and in securing the cannula in place so that communication is unobstructed. Some difficulty may be experienced in passing through the aortic valve, and it is important not to use force, the best results being obtained by teasing the valve orifice over the tip of the cannula.

A silk thread is tied either around the tip of the ventricle or to a small clip attached to the apex. The former procedure is most easily carried out with the heart and cannula empty, and with the cannula inserted through a rubber stopper held firmly by a clamp. The preparation is suspended in a simple chamber made from the upper 7 cm of a Pyrex test tube of 22 mm diameter, with a tightly fitting rubber stopper, the chamber being kept moist with a piece of moistened filter paper adhering by capillarity to the posterior wall. The thread is passed through pulleys to a writing lever. The recording system should be as nearly free from friction and inertia as possible, and with these precautions and proper counterbalancing of the lever, the amplitude of excursion of the writing point on the drum may be adjusted at will. An excursion of 8–9 cm for the maximum amplitude of contraction gives the desired sensitivity to the method. While adjustments are being made, the fluid in the cannula should be changed frequently.

A suitable preparation may under favorable conditions be used over 2–4 days. While the preparation is at room temperature, the fluid should be changed frequently, but it may be left in the icebox overnight without change of fluid and, if still usable, will recover promptly on being brought to room temperature and on change of fluid. Any preparation exposed to solutions or fluids toxic to the heart will, of course, have a shorter period of usefulness.

b) *Solutions of reference.*—The requirements of the solutions of reference are: (1) they must be capable of sustaining the action of the heart; (2) their Ca^{++} concentration must be accurately known; (3) they must approach as nearly as possible the composition of the unknown solutions or biologic fluids with which they are to be matched.

Our earlier observations on biologic fluids were made by analyzing each fluid separately for its inorganic constituents, the solutions of reference being made up on the basis of these analyses. As a result of investigations reported here, we have found it possible, in making routine observations, to use standard series of solutions of reference. It appears that in ordinary conditions the use of these standard solutions of reference does not introduce an error greater than 0.1 mm/l of Ca^{++} in individual determinations, but it should be pointed out that this error may be exceeded in the case of fluids with marked deviation from the normal in electrolyte content and that, in such conditions, particularly when the K content is high, analysis of the unknown fluids may be desirable.

The standard solutions of reference are based on the solution of van Dyke and Hastings (18). They differ from Ringer's and Locke's solutions chiefly in that they are buffered with approximately the $NaHCO_3$ concentration of human serum and that the pH is controlled and the solutions are oxygenated by saturation with an appropriate CO_2-O_2 mixture. They are isotonic with, and have approximately the ionic strength of, mammalian fluids. Phenol red, which does not affect the heart in the concentrations used, serves as a constant check on pH. Nearly all of the solutions are supersaturated with respect to $CaCO_3$, but unless allowed to become too alkaline they will usually be serviceable for 1 day. Any solutions from which precipitation of $CaCO_3$ has begun must be discarded.

PREPARATION OF SOLUTIONS OF REFERENCE.—For routine observations it is convenient to keep in the laboratory basic solutions containing all of the constituents of the solutions of reference except calcium, which must be added shortly before the solutions are to be used. The basic solutions are kept saturated (at room temperature) with a mixture of approximately 4.0% CO_2 in O_2. The concentration of $NaHCO_3$ in these solutions should be adjusted so that when saturated at 25 C with the CO_2-O_2 mixture the pH at 38 C will be between 7.35 and 7.40, so that when observations are made at room temperature the pH of the fluid and of the solutions of reference will be comparable. Mixtures of CO_2 and O_2 may be obtained on specification from commercial sources, but calculations should be based on accurate analyses. Table 1 may be used to facilitate calculation of the $NaHCO_3$ concentration appropriate to the gas mixture used.

TABLE 1.—Concentrations of $NaHCO_3$ Required for pH 7.35 at 38 C when Solutions Are Saturated with CO_2-O_2 Mixture at 25 C and 760 Mm Barometric Pressure

CO_2, %	$NaHCO_3$, Mm/L	154 Mm $NaHCO_3$, Cc/L
3.5	19.2	124.6
4.0	21.9	142.2
4.5	24.8	161.0
5.0	27.4	178.0
5.5	30.1	195.5
6.0	32.9	213.7

The stock and basic solutions may be made up as indicated in Table 2, and the basic solutions saturated at room temperature with the CO_2-O_2 mixture. Saturation should be repeated whenever solutions of reference are to be made.

The solutions of reference are quickly made up each day by pipetting the appropriate amounts of an accurately standardized solution containing approximately 102.7 mm of $CaCl_2$/l. Preparation and standardization of this solution is carried out in the conventional manner; a variation of ±5% in the final concentration will not affect the results significantly, provided the concentration is accurately known. Although quantitative precision requires that the standard solution be measured into a volumetric flask and that the flask be filled to volume with the basic solution, no violence will be done to the results if 100 cc of basic solution is pipetted into a flask and the 1 cc, more or less, of the $CaCl_2$ solution required to give the desired final concentration is then accurately measured into the flask with a graduated pipet. For observations on normal serum or cerebrospinal fluid a range of 0.8–1.5 mm/l of calcium, at intervals of 0.1 mm/l, in the solutions of reference, will usually be adequate.

TABLE 2.—Composition of Stock Solutions Isotonic with Each Other and with Mammalian Fluids, and of Basic Solutions, Assuming Gas Mixture of 4.0% CO_2 in O_2*

Salt	Stock Solutions		Basic Solutions	
	Mm/L	G/L	Mm/L	Cc. Stock Sol/L
NaCl (anhydrous)	154.0	9.00	127.4	827.6
KCl (anhydrous)	154.0	11.48	2.0†	13.0
$NaHCO_3$ (anhydrous)	154.0	12.93	21.9*	142.2
$MgCl_2 \cdot 6H_2O$	102.7	20.88	1.0	9.7
Phenol red		1.00		7.5
Glucose				2.0 g

* For gas mixtures other than 4.0% CO_2, see Table 1.

† This concentration is appropriate to cerebrospinal fluid. We have generally used 5.0 mm/l, or 32.5 cc of stock solution of KCl/l, for comparison with serums on the assumption that all the potassium in serum is ionized.

c) *Technique*.—The heart is usually started with the solution of reference containing 1.2 mm/l of calcium. After adjustments are made and the record is started, the cannula is emptied and refilled with the same solution to determine whether the heart has reached a stable condition. If a change in amplitude occurs, the cannula is emptied and refilled with the same solution, about once a minute, until amplitude is stabilized.

The range of sensitivity is tested by changing the solutions. Change from 1 solution to another is effected by emptying the cannula with a medicine dropper, washing twice with the new solution and filling with the new solution to exactly the same level as before, this level being most conveniently that at which the fluid just reaches the top of the cannula during contraction of the ventricle. The speed of reaction of the heart to a change in Ca^{++}

of not more than 0.2 mm/l is such that equilibrium with the new solution is frequently reached by the time the change of solution is completed.

For each preparation there is a certain minimal concentration of Ca^{++} which will provoke a maximal contraction. The method, therefore, is limited in its usefulness to solutions or fluids which provoke a response less than the maximum. Under favorable conditions the maximal response is not reached until the concentration of Ca^{++} is 1.5 mm/l or higher, but individual preparations vary considerably. Solutions containing less than 0.5 mm/l of Ca^{++} are not favorable to sustained action of the heart and are unsuitable for general use. The most practicable range for observations by this method is 0.5–1.3 mm/l.

After the range of sensitivity is established, the fluid is again changed to 1 with a Ca^{++} concentration expected to be near that of the unknown. In the case of normal serum or cerebrospinal fluid, this is about 1.2 mm/l of Ca^{++}. When equilibrium with this solution is established, a change is made to the unknown. The process is then continued, the known solutions being varied until the unknown is matched and until the sensitivity is again established by demonstrating that the next higher solution will cause an increase and the next lower solution a decrease in amplitude. The concentration of calcium in the known solution which matches the unknown may then be taken as the concentration of Ca^{++} in the unknown. If the unknown falls between 2 known solutions, the concentration of Ca^{++} may be estimated to within 0.5 mm/l. It is desirable to repeat each observation at least twice and preferably 3 times in order to eliminate the influence of momentary fluctuations in the response of the heart.

When the comparisons are being made, particular attention is paid to the height of systolic contraction, since the effect of varying Ca^{++} concentrations is more constant on contraction than on relaxation. Behavior during relaxation, however, serves as a useful check on the condition of the heart and on the uniformity of the conditions under which it is working.

When adequate material is available, any solution which has been in the heart and cannula should be discarded. In the case of limited amounts of biologic material the same fluid may be used repeatedly provided proper precautions are taken to avoid dilution in washing out the cannula. This is done by removing all that is possible of the previous solution before introducing the unknown, a procedure which is simplified by the fact that the heart empties itself almost completely when the cannula is empty. In this way observations have been made on a wide variety of solutions, containing various chemical substances, with little or no difficulty. With such solutions a favorable range of Ca^{++} concentrations can be chosen, and if any toxic effects exist that make the solutions unfit for use, they manifest themselves at once. The method is most accurate and offers the least difficulty with solutions whose composition can be accurately controlled.

d) *Difficulties.*—In the case of certain biologic fluids, difficulties are encountered which may result in loss of accuracy and ease with which observations are made. The chief sources of difficulty are: (1) certain fluids, notably dog serum, are toxic, and the heart survives only limited exposure to them; (2) concentration of Ca^{++} in normal or hypercalcemic fluids may be at or above the upper limit of sensitivity of the heart; (3) if hemolysis has occurred, concentration of potassium in the serum may be too high; (4) most biologic fluids contain substances which exert a pressor effect independently of the Ca^{++} concentration, and this must be differentiated from the Ca^{++} effect.

As a rule, these difficulties can be overcome, but in rare instances no reading can be obtained. Cerebrospinal fluid from man and from dogs has caused relatively little difficulty. As to serum, the least difficulty has been encountered with human material. Rabbit serum is nontoxic but, on account of its high Ca^{++} content, is apt to cause difficulties with the range of sensitivity. Dog serum is almost uniformly toxic and the heart survives exposure to it for only a few minutes.† Beef serum is relatively unfavorable. Pleural and chest fluid and edema fluid of human origin have caused no difficulties. Serum from patients in uremia has not proved toxic, but it is doubtful that the standard solutions of reference are comparable to these serums. Aqueous humor promptly removed from fresh slaughter-house material is favorable. Chicken serum is satisfactory. All biologic material should be used fresh, and there should not be more than a faint tinge of hemolysis. Serum should not stand in contact with cells. Fluctuations in Ca^{++} concentration due to a moderate change in pH are not great, even in protein-containing fluids, but it is desirable to saturate the material with the CO_2-O_2 mixture used for the standard solutions.

Granted nontoxic material, the most serious difficulty is with respect to the range of sensitivity, and a wide variation in behavior is seen in different preparations. Some preparations show a satisfactory range when fresh, others after a few minutes to an hour or so, others only after a night in the icebox. Still others show a satisfactory range in the standard solutions but become insensitive in the higher ranges after exposure to the pressor effects of serum. Seasonal variations and the general condition of the frogs, plus individual variations, seem to play the greatest part in determining the range of sensitivity. The following suggestions may prove useful if this difficulty is encountered: (1) select healthy frogs; (2) have patience in the hope that the range will improve; (3) expose the heart for brief periods to higher concentrations (2.0 mm l) of calcium; (4) discard the preparation in favor of a new one if it proves unsuitable. The sensitivity in the upper ranges may also be improved by lowering the temperature at which observations are made (see discussion of temperature, p. 77). In general, we have found it possible to secure a range of sensitivity satisfactory with normal serum except for a month or 2 just before freshly caught frogs are available in the spring, when the greatest difficulties with the range of sensitivity are encountered.

---

† This high toxicity has been obviated by use of sodium polyanethol-sulfonate (Liquoid-Roche, obtained from Hoffmann-LaRoche, Inc., Nutley, N. J.) as an anticoagulant. By this means, plasma is obtained in which the calcium ion concentration remains unchanged and which is nontoxic for the frog's heart.

The difficulty with pressor effects, uncomplicated by difficulty with the range of sensitivity, is much more easily overcome. The effects of pressor substances so far observed differ from the effect of increasing Ca^{++} mainly in the speed with which the effect is produced and in the time necessary for it to disappear. The effect of a change in Ca^{++} concentration is very rapid in both directions, the new equilibrium being frequently reached, as already observed, by the time the change of solutions is completed. On the other hand, the maximal effect of a pressor substance may be obtained only after 2 or 3 min and wears off very gradually when the pressor substance is removed. When in the course of an observation the effect of a pressor substance is seen, it is necessary only to wait until it reaches its maximum and then to proceed with comparisons at the new level. That this procedure is valid has been shown by experiments in which epinephrine has been added to artificially prepared solutions.

Subject to these limitations, reasonably satisfactory readings may be obtained with serum, and more satisfactory observations with cerebrospinal fluid and other protein-free or protein-poor fluids. The validity of the method in the case of serum has necessarily been based largely on the collateral evidence obtained in investigations the results of which are to be published in detail.

Sensitivity.—Within its particular range, every heart examined has been sensitive to changes in Ca^{++} concentrations. In the lower ranges, a change of 0.1 mm l in Ca^{++} concentration may result in a change of 10 mm or more in the height of the record, but as the upper limit of the range of sensitivity is approached, the same change in Ca^{++} concentration may produce a change of not more than 2–3 mm in the record. Under favorable conditions and with Ca^{++} concentrations at about 1.0 mm/l, the sensitivity, and hence the theoretical accuracy of the method, is to within ±0.1 mm/l of Ca^{++}, this sensitivity increasing with lower concentrations, so that there is a sensitivity and theoretical accuracy of ±10 per cent over the available range. Under the most favorable conditions, involving use of solutions of identical composition except for Ca^{++} concentration, a sensitivity to differences of Ca^{++} concentrations of 0.025 mm/l, or 1 mg/l, has been demonstrated.

Temperature.—From the standpoint of the preparation, the most satisfactory range of temperature is 15–25 C. Our observations have been made, as a rule, at room temperature, ordinarily between 22 and 25 C. As the temperature rises above 25 C, the upper limit of the range of sensitivity tends to fall, and at about 30–32 C the heart usually stops beating. At 15 C, although the rate is slowed, the sensitivity in the upper ranges is usually increased.

Variations in solutions of reference.—The solutions of reference described are, as stated, based on the average inorganic composition of the biologic fluids with which they are compared. Their composition represents our best judgment, based on careful studies of the effect of varying each constituent independently, and it appears that the use of these standard solutions of reference does not, under ordinary conditions, introduce errors of more than 0.1 mm/l in the estimation of Ca^{++} in biologic fluids of approximately normal inorganic composition.

EVALUATION

Under favorable conditions the method has proved to be workable and reliable. The chief difficulty is that it is tedious and requires considerable skill and judgment on the part of the observer. Its usefulness is subject to seasonal variation in the sensitivity of frog heart preparations. It is not adaptable to routine use in the clinical laboratory.

For the purposes of the clinical laboratory, the nomogram (Fig. 2) of McLean and Hastings (13), which requires only chemical determination of the total calcium and total protein gives results which appear to be at least as reliable as those obtained by direct observation by use of the frog's heart. Calculation of Ca^{++} concentrations by the nomographic method may be complicated or even invalidated by abnormal patterns of plasma proteins, since some of the globulin fractions vary greatly in their calcium-binding power (5), or by abnormal concentrations of phosphatids in the plasma (4). Under such conditions, however, estimation of Ca^{++} by the frog heart method is not interfered with.

On the positive side it may be said that the method is self-calibrating and that, with solutions which correspond closely in chemical composition to the reference solutions, results with a high degree of accuracy can be obtained. The dissociation constants of calcium, magnesium and strontium citrates were determined by this method (7), and these constants were subsequently shown by other methods (9) to be reasonably accurate.

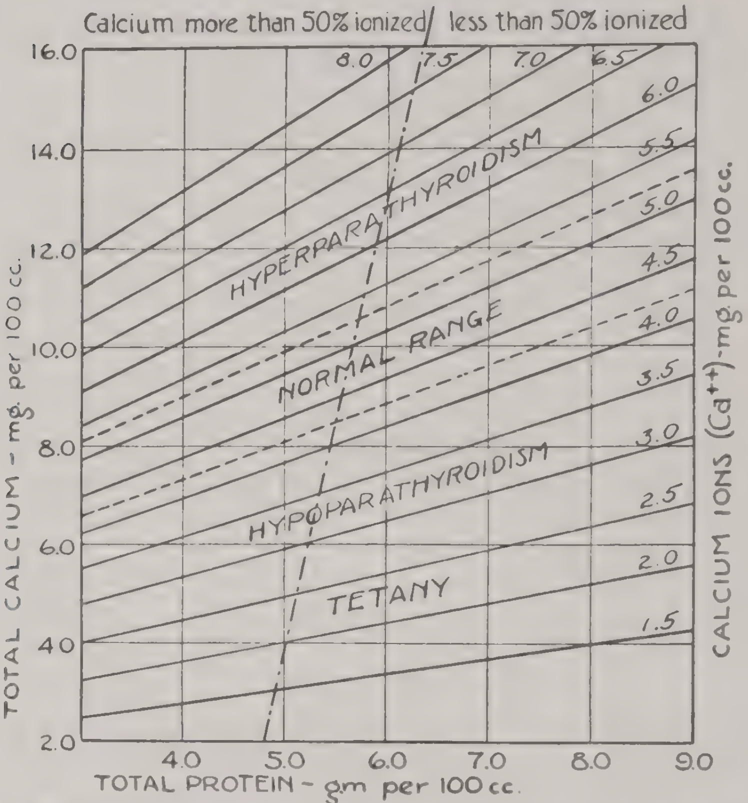

Fig. 2.—Nomogram for calculation of Ca^{++} concentration from total protein and total calcium of serum or plasma. (From McLean and Hastings (13).)

An accurate physical or chemical method applicable to biologic fluids for the determination of calcium ion concentration is much to be desired. Until such a method is available the method here described, difficult as it is, will be found useful for certain purposes.

Note.—This section was reviewed by A. B. Hastings.

REFERENCES

1. Clark, A. J.: The action of ions and of lipoids on the frog's heart, J. Physiol. 47: 66–107, 1913.

2. Clark, A. J.; Percival, G. H., and Stewart, C. P.: Action of calcium ions on the frog's heart, J. Physiol. 66: 346–355, 1928.
3. Compere, E. L.: McLean, F. C., and Hastings, A. B.: State of calcium in the fluids of the body: II. Calcium in the blood in rickets, Am. J. Dis. Child. 50: 77–83, July, 1935.
4. Drinker, N., and Zinsser, H. H.: The equilibrium between calcium and cephalin in various systems, J. Biol. Chem. 148: 187–196, 1943.
5. Drinker, N.: Green, A. A., and Hastings, A. B.: Equilibria between calcium and purified globulins, J. Biol. Chem. 131: 641–647, 1939.
6. Günther, F., and Huebner, W.: Calcium wirkung und Calciumionen, Klin. Wchnschr. 3: 789–790, 1924.
7. Hastings, A. B., *et al.*: The ionization of calcium, magnesium and strontium citrates, J. Biol. Chem. 107: 351–370, October, 1934.
8. Joseph, N. R.: Interaction of amino acids and salts: III. The determination of the activities of calcium, barium, and strontium chloride in amino acid solutions by means of electrodes of the third kind, J. Biol. Chem. 130: 203–217, September, 1939.
9. Joseph, N. R.: The dissociation constants of organic calcium complexes, J. Biol. Chem. 164: 529–541, August, 1946.
10. Klinke, K.: Neuere Ergebnisse der Calcium-forschung, Ergebn. d. Physiol. 26: 235–304, 1928.
11. McLean, F. C.; Barnes, B. O., and Hastings, A. B.: The relation of the parathyroid hormone to the state of calcium in the blood, Am. J. Physiol. 113: 141–149, September, 1935.
12. McLean, F. C., and Hastings, A. B.: A biological method for the estimation of calcium ion concentration, J. Biol. Chem. 107: 337–350, October, 1934.
13. McLean, F. C., and Hastings, A. B.: Clinical estimation and significance of calcium-ion concentrations in the blood, Am. J. M. Sc. 189: 601–612, May, 1935.
14. McLean, F. C., and Hastings, A. B.: The state of calcium in the fluids of the body: I. The conditions affecting the ionization of calcium, J. Biol. Chem. 108: 285–322, January, 1935.
15. McLean, F. C., and Hinrichs, M. A.: The formation and behavior of colloidal calcium phosphate in the blood, Am. J. Physiol. 121: 488 494, February, 1938.
16. Straub, W.: Verhandl. d. deutsch. Gesellsch. f. Naturforsch. u. Aerzt 84: 192–214, September, 1912.
17. Trendelenburg, P., and Goebel, W.: Tetanie nach Entfernung der Eipthelkörperchen und Calciummangel im Blute, Arch. f. exper. Path. u Pharmakol. 89: 171–199, 1921.
18. Van Dyke, H. B., and Hastings, A. B.: The response of smooth muscle in different ionic environments, Am. J. Physiol. 83: 563–577, January, 1928.

Determinations with Flame Photometer

PAULINE M. HALD, *Yale University*

The development of the flame photometer marked a great advance in procedures for the measurement of sodium and potassium for both investigators and clinicians. Because commercial production was retarded and early instruments were not completely satisfactory, individual investigators found it necessary to build their own instruments or to remodel available ones. From the reports of the experiences of these workers, which have been circulated chiefly by personal communication, conflicting opinions and some misapprehensions have arisen. To evaluate the present position of the flame photometer and its application to medical research, it seems desirable to present briefly the available information, with emphasis on (*A*) scope of the instrument (p. 89), (*B*) skill and knowledge required for its successful operation (p. 89), (*C*) accuracy of the measurements which can be achieved (p. 90), (*D*) selection of an instrument from the various types available (p. 90), and (*E*) specific directions for its operation (p. 91).

Since sodium and potassium occur in body fluids in relatively constant concentrations, small variations have distinct significance. To be satisfactory, therefore, an analytical method must have sufficient precision to distinguish such small changes. Conventional chemical methods are time-consuming and require relatively large volumes of material. The frequency of measurements of sodium and potassium has therefore been limited, and studies on infants and small animals have been difficult, if not impossible. The time required for completion of analyses has prevented their use by clinicians for the direction of therapy. The direct reading flame photometer has made it possible to obtain prompt, accurate results with only small amounts of material. Within a half-hour it is possible to measure both sodium and potassium concentrations in as little as 1 ml of serum with an accuracy, under optimal conditions, at least equal to that of accepted chemical methods.

In industrial chemistry, for which it was designed, the flame photometer was at once accepted as a successful analytical instrument. Samples in aqueous solution, rarely limited to small volumes and varying widely in concentration, were readily analyzed. Biologic materials offered particular obstacles. The necessity for precise measurement in low concentrations in the presence of proteins complicated the analysis of serum and whole blood. The complexity and variability of the composition of urine, feces, food and tissues presented altogether different problems. It became evident quite early that the instrument required more precise evaluation and regulation to meet the demands of biologic chemistry. In this basic evaluation numerous unexpected variables were encountered which led to disappointment and disparaging reports, misconceptions and apprehensions. Verbal reports of the performance of a given instrument were often contradictory. Frequently, expectations were too great. Instruments were purchased under the misconception that satisfactory analytical results could be obtained after a day or two of calibration and experience. Such optimism was destined to disappointment and delay. To some, the difficulty of operation seemed great enough to compel a return to chemical methods of analysis (17). As experience has revealed the reasons for the numerous difficulties, confidence in the reliability of the flame photometer for measurement of sodium and potassium in biologic materials has been established.

THE INSTRUMENT

The direct reading flame photometer represents a step in the logical development of the Lundegardh method of spectrophotometric analysis (12). In comparison with the spectrograph the flame photometer has the particular advantage of simplicity. It is, however, far more limited in its applicability (10).

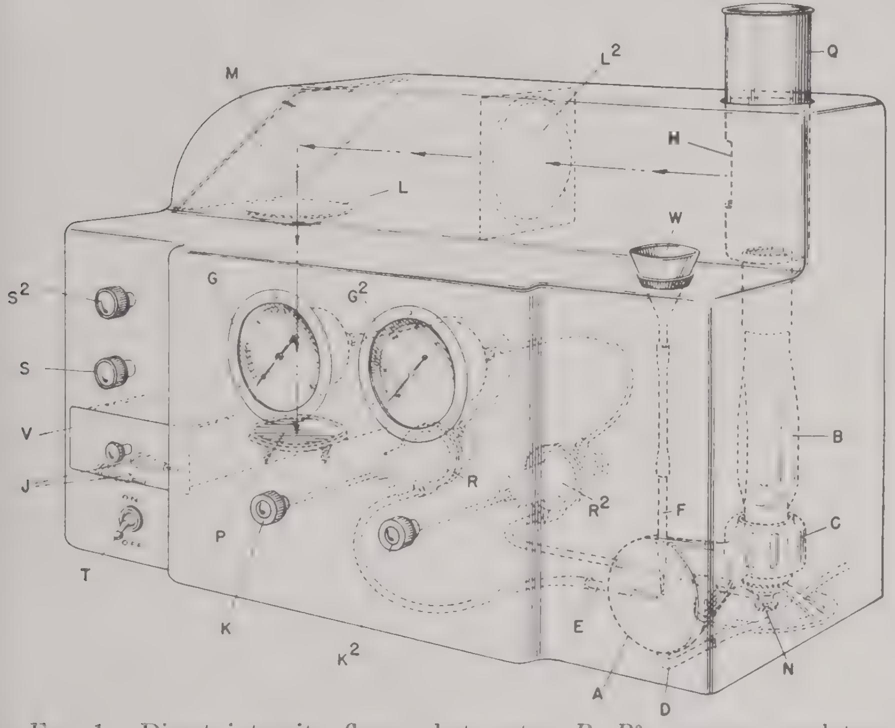

FIG. 1.—Direct intensity flame photometer. R, R^2, pressure regulators; K, K^2, control knobs for pressure; G, G^2, pressure gauges for gas and air; E, air jet of atomizer; F, fluid tube atomizer; W, funnel; A, atomizing chambers; D, drain; C, mixing chamber; B, burner; N, needle valve; H, window in chimney; Q, chimney; L, L^2, lenses; M, mirror; V, filter system; P, barrier layer photocell; T, toggle switch; J, galvanometer jack; S, S^2, sensitivity adjustment, coarse and fine. (Figs. 1–3 from Barnes *et al.* (2) and Berry *et al.* (4).)

The principle was described by Barnes, Richardson, Berry and Hood (2) in 1945 (Fig. 1). In their original instrument the solution to be analyzed is discharged, by the force of compressed air, through an atomizer in the form of a mist. This is drawn into the flame, where it is ignited. The light produced by the combustion, which is characteristic of the element to be measured, is freed from other radiation by a selective filter. The filtered light impinges on a barrier layer photocell and the electrical energy produced is measured by a sensitive galvanometer. The photometer is calibrated against solutions which contain known amounts of the element to be measured. This simple, direct reading instrument has yielded a large body of satisfactory data. Such performance has been possible because the variable factors of the instrument have been well controlled and the biologic material to be analyzed rarely contains interfering ions in great enough concentration to affect seriously the determinations in the highly diluted samples which are used. The direct intensity method requires absolutely stable and unvarying conditions for satisfactory performance. Such conditions are, however, by no means impossible to achieve and require but little more precision in adjustment than is necessary to secure satisfactory performance from instruments that do not operate on the direct reading principle.

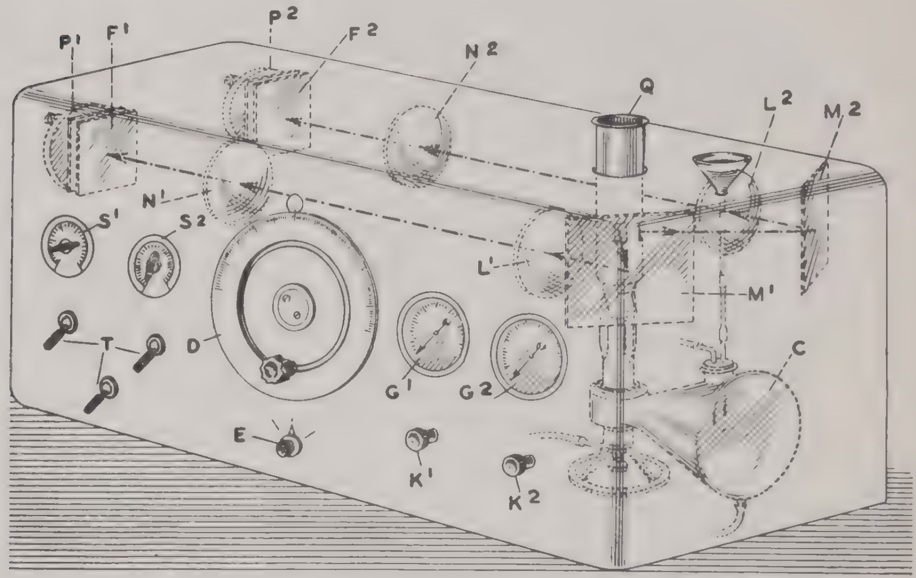

Fig. 2.—Internal standard flame photometer. *Q*, chimney; M^1, M^2, mirrors; L^1, L^2, N^1, N^2, Fresnel lenses; F^1, sodium or potassium filters; F^2, lithium filters; P^1, P^2, barrier layer photocells; *C*, atomizing chamber; G^1, G^2, pressure gauges for gas and air; K^1, K^2, gas and air regulator knobs; *D*, main potentiometer dial; *E*, compensating rheostat; *T*, toggle switches; S^1, S^2, sensitivity adjustment, coarse and fine.

An improved method of flame photometry was later proposed by Berry, Chappell and Barnes (4) (Figs. 2 and 3). In this method light intensity ratios rather than absolute light intensities are measured by utilizing an internal standard principle similar to that employed in spectral analysis. A fixed quantity of some element (internal standard) not ordinarily found in the sample is added before the sample is analyzed. Since it is advantageous to use an element that has excitation characteristics similar to those of the element to be determined, lithium was selected. Upon excitation both elements emit light. The ratio of the intensities of these 2 emissions is measured. Since slight fluctuations in the discharge of the atomizer or in the vigor of the flame affect the ignition of both elements to approximately the same extent, the ratio is not appreciably altered. Furthermore, the effects of viscosity and mutual interference from the presence of other ions are minimized by this method. According to Berliner (3), so long as the ratio of the concentration of the unknown element to that of lithium is precise, the final volume of dilution can vary by as much as 5% without affecting the reading. This is a great advantage when large numbers of samples must be diluted. Perhaps the greatest advantage of the internal standard instruments is the convenience with which they can be read. The dial of a potentiometer is rotated until a null point is reached on the recording instrument. The reading on the dial can then be noted at the operator's con-

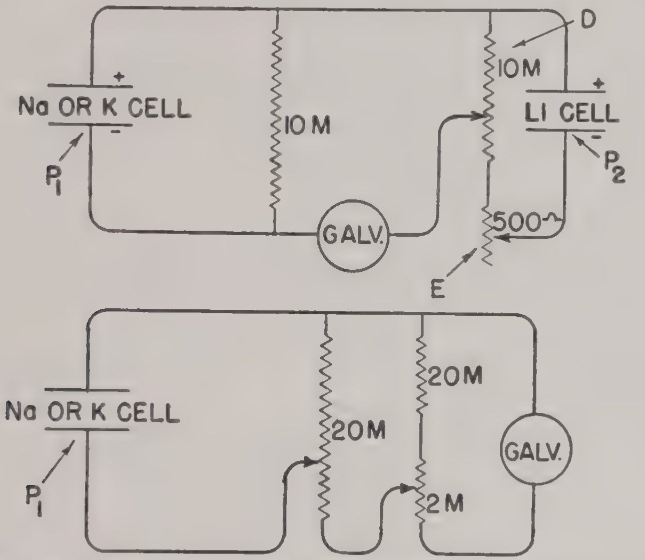

FIG. 3.—Circuits of internal standard flame photometer. *Above*, ratio circuit; *below*, absolute circuit.

venience. This eliminates the necessity of estimating it from the fleeting deflection of a galvanometer "spot," which is necessary in the direct intensity method.

VARIABLE FACTORS

The operation of all flame photometers, of whatever design, depends on integration of a number of variables peculiar not only to a given type of instrument but to each particular instrument and to the conditions under which it is presently being operated. Among the variables which especially require consideration are: (1) the method of introducing the sample, (2) atomizer, (3) flame, (4) filters, (5) photocells, (6) cabinet, (7) location of the instrument, (8) interferences, and (9) preparation of the sample.

1. *Method of introducing sample.*—The sample may be introduced by either gravity or suction. In the former procedure the solution to be analyzed is poured directly into a funnel placed above and connected with the atomizer. In the suction method there extends down from the bottom of the atomizer a fine tip through which the solution is drawn into the atomizer by suction from a beaker or other small receptacle. Although the latter method is preferred by some, it requires an additional vessel for each sample and affords greater opportunity for evaporation and contamination than does the gravity method.

2. *Atomizer.*—This is probably the most critical feature of every instrument. Unless the mist carried to the flame is steady and composed of particles of nearly uniform size, the rate of emission of light will be unstable. The resulting fluctuations will be reflected in the galvanometer readings. For analysis of biologic materials, particularly serum and other protein-containing fluids, the atomizer must have certain characteristics which are not essential for analysis of simple salt solutions. If it is not rapidly self-cleaning, after a few samples have been introduced irregular flow will cause much difficulty. Diminishing the size of the orifice of an atomizer permits economy of material but is likely to lead to intolerable plugging and consequent poor operation.

After many designs of glass atomizers had been discarded, Barnes *et al.* (2) devised the one originally described from 2 hypodermic needles. With slight modifications (1, 7), this type has been used extensively for both direct intensity and internal standard instruments. It is simple, inexpensive, easily made or adjusted, durable and readily cleaned. A straight lumbar puncture needle is used for delivery of the sample; it lacks the shoulders which are present in hypodermic or intravenous needles. The opening, therefore, is large enough to permit insertion of a long stilet from above to dislodge occasional particles or air bubbles which retard the rate of flow. The compressed air is admitted through a regular hypodermic needle in such a way as to send the spray of mist in a direction at right angles to that of the straight needle. The paint spray type of atomizer, made of stainless steel, is preferred by many. This type, when made entirely of hard rubber, may be used for samples which would be destructive to metal.

Glass atomizers are also used. The chief disadvantages are fragility and lack of uniformity. Even a skilled glass blower cannot reproduce his work exactly. Repair or replacement of a glass atomizer therefore may necessitate readjustment of several other variable factors before operation of an instrument can be resumed.

To produce a uniform spray, an atomizer must be kept scrupulously clean. Protein deposited during analyses of serum and phosphates or other salts deposited during analyses of urine tend to change the rate of flow through any atomizer. Tiny particles occasionally plug the small orifice or air bubbles interrupt the flow. The atomizer should be so constructed that cleaning can be done with a minimum of delay in operation. Deposited precipitates can be removed only by soaking in a solvent appropriately selected to dissolve either protein or salts, as the case may be. Some operators keep a solvent in the atomizer system at all times between series of analyses; others prefer to change units frequently, soaking the atomizers periodically in a suitable solvent or solvents. Solid particles or air bubbles can be dislodged by stilets.

ATOMIZING CHAMBER.—A chamber is incorporated in most instruments to house the atomizer and convey the mist into the burner. Although of some importance, it is secondary to the efficiency of the atomizer and quality of the flame. It must, however, be so devised that only fine, uniform droplets reach the flame. In most chambers the larger particles are condensed on the walls and subsequently led off through a drain. Though loss of solution by this means may appear to be wasteful, it is usually not serious with respect to the original sample because the dilutions used are large. Nevertheless, to permit use of small volumes of solution, some instruments have a cylindrical glass chamber equipped with an electrically heated cover. This prevents condensation on the chamber walls, so that all of the sample reaches the burner. Some chambers have an open collar, encircling the base of the burner, in which the mist is mixed with gas and room air. Others are sealed to a tube which leads directly into the burner so that the only supply of air which reaches the flame comes from the compressor. This type requires a greater supply of compressed air.

Weichselbaum and Varney (20), after a searching study, abandoned the chamber. They designed a combined atomizer and burner which introduces a constant amount of the atomized solution per unit of time into a flame of constant thermal output. They believe the chamber contributes more or less uncontrollable factors, among which are the temperature of the atomizing chamber itself and electrostatic effects due to variations of charge on its walls.

3. *Flame*—The flame depends for its success on proper regulation of the delivery of gas, air, oxygen or whatever combination of these is required to give constant thermal conditions with a given burner. The importance of a constant, satisfactory flame cannot be overemphasized. This requires reliable instruments for regulation of the flow of both gas and air. The appearance of the flame is an important guide to its performance. Some instruments have a mirror permanently mounted in such a position that the operator can observe the flame at all times. The flame should be steady, free from pulsation and clear blue, free from streaks of yellow or green. Some experimentation, using various combinations of gas and air pressures for the construction of calibration curves, is necessary to define the best possible flame conditions.

The gas burned must be constant in composition as well as pressure. In some areas natural gas has proved satisfactory. Manufactured city gas is usually too variable. "Bottled" gas of the propane type is most commonly used. The temperature of the propane flame is particularly suitable for measurement of sodium, potassium and lithium. At the higher temperatures produced by oxygen and gas, or oxygen, air and gas, calcium can also be measured, but the danger of exciting mutually interfering elements is incurred. To compensate for this it is necessary to introduce involved procedures which sacrifice the simplicity of operation, which is the chief virtue of direct reading instruments (10). Acetylene, which has been recommended for determination of elements other than sodium, potassium and lithium (16), yields a temperature high enough to cause serious interference. With this gas, indeed, under most conditions, even sodium causes mutual interference with potassium.

Compressed air is customarily used to produce the spray which eventually reaches the flame. Pressures of 10–30 lb/sq in. are usually recommended. Unless the laboratory air supply can be relied on to maintain such pressures, a compressor is necessary. The air must be dry and clean. It may, therefore, be necessary to pass it through a filter, and sometimes through a drying chamber, to remove impurities and moisture.

4. *Light filters.*—Usually simple filters are used to isolate the desired light in the regions of the spectrum suitable to the element to be measured. In some instruments monochromators are used.

These devices necessitate the introduction of extrasensitive means of detection which detract from cheapness and simplicity (10).

5. *Photocells.*—The barrier layer cell has the great advantages of simplicity, broad spectral response, steadiness and reproducibility. In addition, it requires a relatively simple electrical circuit, a highly desirable feature in an instrument designed for use in biologic and hospital laboratories. Since its emission energy is low, its output must be measured by a sensitive galvanometer.

True photoelectric cells (phototubes) require special power supplies and amplifiers, with far more complex electrical circuits that can be serviced only by experts.

6. *Cabinet.*—Because the cabinet must accommodate all the parts of the instrument in their particular relationships, it is usually large and contains much dead space. In addition to its function as an enclosing framework, it is also intended to maintain stable thermal conditions. Since they respond irregularly to heat, barrier layer cells must be protected from high temperatures. They should, therefore, be placed at some distance from the flame. Some cabinets are constructed of wood lined with asbestos to insulate the cells from the heat of the burner. Far more are made from metal which, being a good conductor, dissipates the heat into the surrounding atmosphere.

7. *Location.*—The location of the flame photometer should be given the same consideration as that of a fine analytical balance. Thermal conditions in the room should be stable. Drafts and intermittently operating radiators should be avoided. If possible, the instrument should be placed in a hood or be equipped with a flue to carry off the incinerated materials to prevent them from settling in the room to contaminate subsequent analyses. A portable table to hold the bottles and flasks containing standard solutions and diluted samples is also desirable. If these containers are placed on the counter near the flame photometer, the dried residue of any spilled solution may easily find its way into the flame. Even with the greatest precautions, sodium and potassium are so ubiquitous that momentary fluctuations of the recording instrument may be induced by motions of individuals in the room or even across the doorway. Particularly when low concentrations of the elements are being measured, traffic should be reduced to a minimum. The best results are usually obtained when the operator is alone in the room with the instrument. Tobacco smoke must be excluded because of its contaminative effect in the determination of potassium.

8. *Interferences.*—The term "interference" in flame photometry has been applied to every variety of disturbing conditions: variations in the atomizer or burner, viscosity factors and contamination

from any source. It should be reserved to indicate the influence of foreign elements in solution on the emission of the element being measured. Interferences in this technical sense constitute a major problem of flame photometry. They may be divided into 2 categories (6). The first is spectral interference which arises from presence of an element with a strong line or band in close proximity to or overlapping that of the element to be measured; e.g., in analyses of serum, sodium strongly interferes with calcium. The second is mutual interference which arises when an element which emits no interfering spectrum affects the intensity of the spectral lines of the element to be measured; e.g., in the hot acetylene flame, sodium enhances the light of the potassium flame.

The presence of such interferences often requires introduction of complex procedures which detract from the simplicity of the direct reading instrument and create new problems and difficulties. To obviate or minimize interferences various expedients are used.

a) The interfering ion may be removed chemically before the analysis. For example, calcium may be precipitated with ammonium oxalate, isolated and redissolved before it is introduced into the photometer. While this and similar expedients are feasible, the resulting procedure often has little or no advantage over conventional chemical methods.

b) Compounded standard solutions may be used to establish calibration curves. These should contain concentrations of the interfering substances approximating closely those in the unknown solution. For serum, because of its relatively uniform composition, this is quite possible, but for such complex and variable solutions as urine, feces and tissue extracts it is altogether impracticable.

c) Flame "background" (14) may be determined by separate evaluation of the interfering ions in each sample. A solution of similar concentration with respect to the interfering element is read to determine the blank correction. When this factor is not too great, a proper correction may be applied.

d) The principle of the internal standard tends to minimize these interferences. Within limits it eliminates mutual interference.

e) High dilutions reduce the effect of interferences.

f) A relatively low flame temperature, like that of propane, does not excite many interfering ions.

9. *Preparation of sample.*—The most desirable method of preparation of the sample is by simple dilution with water. With liquids such as serum, whole blood and urine this is possible. Solid or semisolid material such as food, feces and tissues must be ashed or extracted by some accepted procedure before proper dilution can be made.

In general it is advisable to dilute to the lowest concentration which lies within the range of optimal performance of the instrument. There are several advantages to be gained: (*a*) material is economized; (*b*) the effects of viscosity and/or mutual interferences from foreign ions are minimized, and (*c*) the curve tends to become more nearly linear. The chief disadvantage is that the danger of contamination from reaction with bottles, touching stoppers, glassware which is not scrupulously clean, and room dust is magnified. Higher concentrations not only sacrifice the previously mentioned advantages but tend to clog the atomizer and burner.

A. SCOPE

The flame photometer is widely used for Na and K determinations in various biologic materials. These can be done accurately, rapidly and simply. Great interest has been evinced in the possibility of determining additional cations, especially Ca and Mg. As far as serum is concerned, this interest has not been rewarded in practical results. While it has been reported by a few workers that serum Ca can be measured, this has not been generally confirmed with any instrument and no extensive data with supporting control analyses have been presented. A forthcoming type of instrument shows great promise that serum Ca in dilute solution may be determined. It is not yet available and its performance in the hands of several analysts will be necessary for its proper evaluation. Unfortunately, even this instrument is not yet applicable to the measurement of Mg in serum (19).

In urine and other material where Ca is present in higher concentrations, better results may be expected. Satisfactory recoveries in urine have been reported. A hot flame and proper corrections for spectral interference are required (6). It is claimed that the forthcoming instrument already mentioned is suitable for measurement of Mg in urine after its isolation as the 8-hydro-oxyquinoline (19).

B. SUPERVISION

A flame photometer must be set up and its operation supervised by a trained, quantitative analytical chemist. This individual must, himself, experiment with variations of each of the critical components (atomizer, flame, air pressure, gas pressure, etc.) in order that optimal conditions of performance may be recognized and reproduced.

From this experience a proper procedure must be planned so that an untrained or semiskilled person can be taught to operate the instrument. Rigid controls must be set up to enable the operator to become aware of any deviation from optimal conditions.

When such deviation does occur, a conscientious and observing technician will report the departure immediately to the person in charge. It has proved to be entirely practical to have as many as 14 different operators assigned to 1 instrument keeping it in almost constant use. With proper controls and adequate instruction, the supervisor can be quite free and need be called on only in an advisory capacity.

C. ACCURACY

To detect small changes of concentration in serum of Na and K, determinations must be accurate to ±1.5% for Na and ±5% for K at the levels at which they occur. Greater accuracy, while highly desirable, does not add greatly to the value of these measurements. Many photometers, however, particularly those using the internal standard principle, are capable of precision of less than 1% for both Na and K.

Accuracy is determined by 2 procedures: (1) recovery of a known amount of Na or K in a solution which contains the various inorganic salts present in serum at approximately their respective concentrations permits evaluation of the effect of interfering ions, and (2) repeated determinations on given samples which have been diluted (each from the original material) to several concentrations measure the effects of viscosity.

More complex materials—food, feces, tissues, etc.—may give less accurate values, depending on the method of preparation. However, when good blending is achieved, the chief deterrent to accurate recovery is the effect produced by interfering ions.

Precision of performance should not be confused with accuracy. Some instruments appear to exhibit very stable conditions of operation and readings are exactly reproducible. However, this effect may be the result of damping the recording instrument. The precision which results, and which is considered highly desirable, is often mistaken for accuracy. Frequently, a less precise (more unstable needle deflection) but more sensitive instrument is capable of greater accuracy.

D. SELECTION OF INSTRUMENT

The selection of a flame photometer should depend on the purpose for which it is to be used. Sodium and K can be measured with simplicity of treatment on small amounts of material (less than 1 ml. for duplicates of both in serum) with an accuracy of less than 1% with the type of flame photometer which is least expensive to purchase and least complex to operate and maintain. The biologic or hospital laboratory, where Na and K analyses are desired, should not find selection difficult.

A recommendation of an instrument by one or more accredited medical laboratories is the best guide in making a choice. The distributor of any instrument should be able and willing to supply such references if the instrument is held in the proper high regard. Direct communication should be established with the person responsible for the performance of the instrument and, if possible, the instrument should be seen in operation. A study of the manual supplied with an instrument will reveal from the circuit diagram whether the electrical circuit is too complex for repair and maintenance by the local personnel. If it appears to be so complex that the instrument will have to be returned to the factory for adjustments, delays in operation must be provided for.

In many research laboratories where investigation depends on more complex analyses, members of the research staff are qualified to adjust and maintain the more intricate instruments. From their knowledge and background they will of necessity choose the instrument which appears most promising for the execution of their particular investigative work. Complexity of electrical circuits and problems of spectral and mutual interferences will be accepted as a part of the studies, if necessary.

Interest in further development of the flame photometer is keen and widespread. Since it is a new instrument, development and improvement are expected. Serum Ca determinations will be possible soon, it is claimed. However, it appears unlikely that any single instrument will determine Na, K and Ca with the same convenience and simplicity that are now possible for the first 2. An instrument which will determine serum Mg has not been described.

E. GENERAL TECHNICAL PROCEDURES

Plans for the operation of a flame photometer should provide for several weeks of intensive work with it by the person who is ultimately to be responsible for its operation and maintenance. This period of orientation is necessary to become familiar with the variable factors and their adjustments. A similar period is required not only for the first instrument which is operated but for every instrument which is used subsequently. During this period of constant observation, different concentration ranges can be tried.

Flame photometers are designed to give accurate results within a limited concentration range. The range selected by the manufacturer applies usually to concentrations which will be appropriate for analysis of serum. The need for the greatest accuracy and at the same time the greatest economy of material is demanded in analysis of serum. Therefore the instrument built for the biological chemist operates best in the very low concentration ranges.

Materials which are far more highly concentrated with respect to Na or K require greater dilutions to bring them within the optimal range. To obviate the necessity for excessively high dilutions, Wallace (18) has interposed a neutral density filter between flame and photocell. By means of this device, which can be made of x-ray film, the concentration range may be changed by approximately 50% without disturbing the variable factors of the instrument in any way. It has been proposed that a more suitable device could be designed which would be adjustable and thereby cut down the amount of light transmitted to the cell to a variable degree. By this means far greater selectivity of concentration range would be possible.

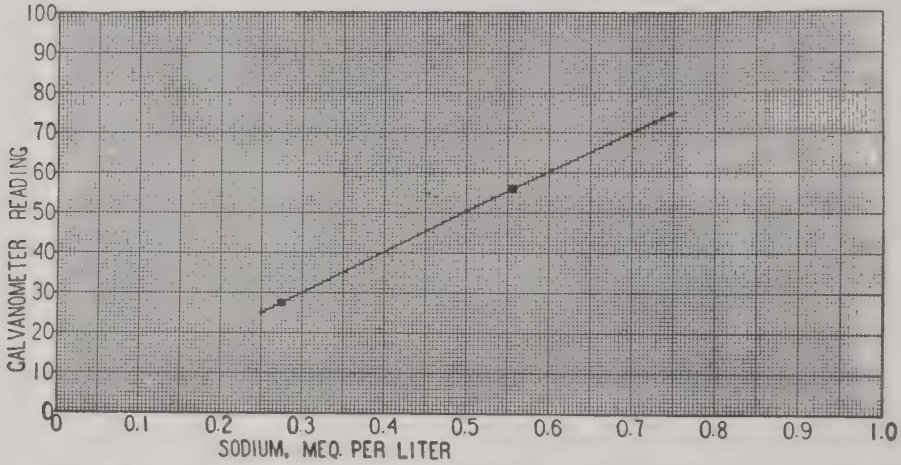

FIG. 4.—Calibration curve for sodium, direct intensity method. Solid dot = standard; solid square = "artificial" serum.

Various adjustments of air and gas pressures, potentiometer settings, effects of drafts or air currents, changes in sunlight in the room and other variables peculiar to the selected location (p. 87) must be related and co-ordinated. To provide the maximal stability and accuracy of which the instrument is capable, there is no alternative. The flame photometer has not "been perfected to the point that it can be accepted in the routine clinical laboratory as is the colorimeter. Further advances must be made before this status is reached" (21).

Readings are made by interpolation on a calibration curve constructed from analyses of standard solutions (see Fig. 4). For construction of such curves, co-ordinate paper ruled 10 squares to the inch is desirable. Galvanometer readings are plotted on the ordinate scale, concentrations of the element on the abscissa. The curve is constructed from readings obtained with a series of standard solutions of NaCl or KCl of suitable concentrations. Four or more points must be used to establish a curve because the relation of readings to concentrations is seldom rectilinear. The curve is prepared to cover a given concentration range, selected to suit the conditions of the analysis under consideration. It will be constant and reproducible only if the flow of solution through the atomizer, the air pressure and the gas pressure are properly related. A small deviation may invalidate the reading. Mere resetting of the galvanometer will not correct such an error. A drifting curve indicates changing conditions, under which reliable measurements cannot be made. It is necessary, therefore, to construct a curve for each set of analyses. Even in the course of such a set of analyses it must be checked. If it does not check it must be reconstructed. Unless this procedure is rigidly followed, results within the limits of error reported cannot be expected.

a) *Preparation of solutions.*—Stock standards and the solutions (p. 96) diluted from them should be prepared according to the most accurate quantitative procedure. Chemicals must be of the highest quality reagent grade. Salts must be dried to constant weight before the calculated amounts are weighed. Distilled water must be free from Na and K. All stock and standard solutions should be stored in glass bottles with tightly fitting stoppers. No-Sol-Vit bottles have not been found necessary, but concentrations of solutions have been checked from time to time against freshly prepared solutions to prove that no reaction with the glass has taken place. Some of the solutions had been stored more than a year. This stability might not be maintained with all types of glass and must be established if No-Sol-Vit bottles are not used.

b) *Preparation of material* (Table 1).—*Serum* and *whole blood* are prepared for analysis by the direct intensity method, by simple dilution. Water is used as a diluent for Na and for K also if the dilution is greater than 1:100 so that precipitated globulin offers no obstacle. When the dilution used for K is only 1:25, as in the direct intensity method, 0.9% NaCl solution is used to obviate protein precipitation. A blank analysis must be made to determine the effect of this diluent and a suitable correction applied. *Urine* needs only to be diluted with water. It should be filtered before dilution if gross particles are present. *Food* and *feces* must, of course, be ashed, as must tissues, and the ash extracted, or another suitable method of preparation applied. Both dry ashing with extraction and direct extraction methods are described.

DRY ASHING IN MUFFLE FURNACE (7).—A sample containing 1 or 2 g of a weighed homogeneous mixture of diet or stool is placed in a weighed dish of Vycor glass. Weight is noted and approximately 1 ml of 4N H_2SO_4 is added per g of material. The water is evaporated from the solution, after which the dish containing the charred material is set in a cold muffle furnace and the temperature brought to 500–550 C. After approximately 8 hr a white ash remains. To this ash is added 1 ml of concentrated HCl for each g of food or stool represented. The dish is warmed to facilitate solution of the ash. With the aid of water the mixture is transferred quantitatively to a volumetric flask and diluted to appropriate volume. This diluted extract is filtered, if insoluble material remains, before it is admitted to the flame photometer.

TABLE 1.—PREPARATION OF MATERIALS FOR FLAME PHOTOMETRY

MATERIAL	SPECIAL PREP.	DILUENT	DILUTION	CONC.,* MEQ/L
		SODIUM Direct Intensity and Internal Standard†‡		
Serum	None	Water	1:250 & 1:500	1.00
Whole blood	"	"	1:200 & 1:400	1.00
Urine	"	"	1:40 or as required	2.50
Food	Ash	Acid & water	1 g:25 ml	2.50
Feces	"	" " "	1 g:10 ml	2.50
		POTASSIUM Direct Intensity Method		
Serum	None	Normal saline	1:25 & 1:12.5	1.25
Whole blood	"	Water	1:100	1.25
Urine	"	"	1:20 or as required	2.50
Food	Ash	Acid & water	1 g:50 ml	2.50
Feces	"	" " "	1 g:50 ml	2.50
		Internal Standard†		
Serum	None	Water	1:125 & 1:250	0.06
Whole blood	"	"	1:250 & 1:500	0.25‡
Urine	"	"	as required	0.25
Food	Ash	Acid & water	1 g:500 ml	0.25
Feces	"	" " "	1 g:500 ml	0.25

---

* Concentration of solution which represents full scale deflection.

† All dilutions for internal standard method must contain Li in appropriate concentrations for the particular instrument used.

‡ Neutral density filter used.

NITRIC ACID EXTRACTION (11).—To an aliquot of homogenized mixture, equivalent to approximately 5 g of original material, in a 250 Erlenmeyer flask, 3 ml of concentrated HNO_3 is added. The flask is heated momentarily to the boiling point on a wire gauze and cooled. The contents are transferred quantitatively to a 100 ml flask, diluted to volume and filtered. For analysis in the flame photometer, a suitable aliquot (usually 10 ml) is diluted to 100 ml, with or without Li, as required.

The dilutions of material (see Table 1) must necessarily be varied to bring the concentrations of Na and K into ranges which are optimal for accurate analysis. In serum and whole blood the bases are present in concentrations which are limited and vary over a relatively small range. Consequently it is not difficult to determine the exact dilutions indicated. The concentrations of Na and K in urine, food and feces offer no such limitation in either range or quantity. It is often impossible to predict the concentrations even approximately. However, since the material is usually readily obtained and the concentration range is so extensive, it is neither necessary nor practical to use the sensitivity ranges described here. When 2.5 mEq is represented by full scale deflection, the dilutions listed in Table 1 serve as a general guide. It is unwise to start with a high dilution unless there is some assurance that this is indicated. If material is limited, as in the case of an ash, a second dilution may be resorted to if the first gives too high a reading.

c) *Method of reading.*—When the curve has been defined and verified satisfactorily, the sample to be analyzed is introduced. After the reading has been taken, the 2 points on the curve, between which the reading falls, are again checked. Only if these 2 points have remained stable can the reading be considered valid. If either or both points have varied, the slope of the curve has changed and it is impossible to say at what time the change in the reaction occurred. Small variations in readings of the standards occur so frequently that the procedure known as "bracketing" is recommended. The standard below the unknown in concentration is read, then the unknown and finally the standard of higher concentration, or the order of reading may be reversed. In this manner a curve is determined for each reading. Few of the most experienced workers have found that this method can be dispensed with, when the greatest accuracy is desired. Any tendency to change in the rate of flow through the atomizer, due to particles or air bubbles in, or protein precipitated from, the solution is reflected in a failure of the readings to attain the points originally established. When this situation arises, the delivery needle may be probed with a stilet or washed with a protein solvent. Haemo-Sol, a commercial cleaner, is satisfactory. Since it contains both Na and K, the system must be thoroughly washed with distilled water immediately after its use. It is practical to soak the atomizer in Haemo-Sol when it is not in use. If possible, the detergent should be allowed to flow directly down the drain and should never be sprayed into the burner. Sterox, a nonelectrolyte wetting agent, is not an effective cleaner. Urine and other protein-free solutions leave a deposit of phosphate which can be removed with dilute HCl.

Whenever possible, it is good practice to read a given unknown at 2 different dilutions. In this way small discrepancies in the concentration of the standard solutions, the presence of contaminants or errors in dilution are made evident. Replicate measurements with different concentrations of a given element constitute the most rigid check on the various sources of error. In determining serum Na, dilutions of 1:250 and 1:500 will yield results which agree within 1 to 1.5% when the conditions are optimal.

Serum K offers the greatest challenge to the performance of the instrument, especially when material is limited. When a dilution of 1:25 is used, unless a cell of supersensitivity is available, the readings fall in the lower portion of the scale. Because the reaction is slow, more than 25 ml would be needed to reach a higher point. However, although the reaction is slow, it is quite stable.

Blank determinations are important and are as necessary to detect contaminating or interfering substances as they are in conventional chemical procedures. In the determination of Na, the distilled water used to dilute the sample must be tested, as must the saline solution used as a diluent in the K procedure. When material is ashed, all of the solutions, H_2SO_4, HCl, HNO_2 and distilled water, are possible sources of interference or contamination. A blank determination carried out simultaneously with the analysis of the unknown will indicate whether or not these solutions are pure. Any deflection of the galvanometer from the zero point, established with a current of air, indicates a positive blank.

When serum is analyzed, it is advisable to run a control "artificial serum" solution. This solution should contain the usual inorganic elements present in serum, in the approximate concentrations in which they occur. Sodium, potassium, calcium, magnesium, chloride, sulfate and phosphate should be included. It has been determined by comparison with ashed samples that the viscosity due to protein does not cause an error at the dilutions used for Na. Saline is used for the more concentrated K dilution to avoid the precipitation of globulin which would be caused by dilution with water. Each set of determinations should be controlled by diluting this solution in exactly the same manner as the unknown serum. Any contamination of the diluent or the apparatus will then be evident by failure to recover theoretical amounts. Furthermore, a similar procedure is essential in analyzing any other material in which the presence of interfering ions might prevent accurate recovery.

REAGENTS

Stock standard solutions.—"ARTIFICIAL SERUM." The following amounts are required to make 1 l of solution:

Sodium chloride*	8.073 g		138.0 mEq/l as Na
Potassium chloride*	0.2091 g	2.8 mEq	4.1 mEq/l as K
Potassium-acid-phosphate (KH_2PO_4)*	0.053 g	1.3 mEq	
Calcium chloride†	0.100 g of Ca		5.0 mEq/l as Ca
Magnesium sulfate*	0.1223 g		2.0 mEq/l as Mg

STOCK SODIUM CHLORIDE (100 mEq/l).—Make up 5.845 g NaCl* to 1 l with distilled water.

STOCK POTASSIUM CHLORIDE I (125 mEq/l).—Make up 9.319 g KCl* to 1 l with distilled water.

STOCK POTASSIUM CHLORIDE II (10 mEq/l).—Make up 0.746 g KCl* to 1 l with distilled water; or 80 ml of the above 125 mEq solution may be made up to 1 l.

LITHIUM SULFATE SOLUTION (10 g/l as to Li).—Make up 92.6 g $Li_2SO_4H_2O$* to 1 l. (Biological chemists conventionally express concentration of solution in terms of mass per unit volume. In many other branches of chemistry, parts per million is the preferred term. Since 1 mg/l equals 1 ppm, the amounts in question may be readily converted.)

PHYSIOLOGIC SALINE.—Approximately 9 g of NaCl/l.

Diluted solutions for direct intensity method.—SODIUM STANDARDS.—From the stock standard NaCl solution (100 mEq/l), prepare a series of dilutions containing 0.25, 0.50, 0.75 and 1.00 mEq/l, respectively.

POTASSIUM STANDARDS.—From the stock standard KCl I (125 mEq/l), prepare a series of solutions containing 0.156, 0.313, 0.625, 0.938 and 1.25 mEq/l, respectively.

Diluted solutions for internal standard method.—SODIUM STANDARDS.—From the stock standard solution of NaCl (100 mEq/l) and the stock solution of Li_2SO_4 (10 g Li/l), make up dilutions to suit the selected concentration range.

Example: Na 1.0 mEq/l with Li 100 mg/l. Measure into a 1000 ml volumetric flask 10 ml of stock solution and 10 ml of stock Li_2SO_4 solution. In a similar manner prepare NaCl concentrations of Na of 0.25, 0.50 and 0.75 mEq/l, each containing 100 mg/l of Li.

POTASSIUM STANDARDS.—To stock solution II (10 mEq/l) appropriate amounts of Li from the stock Li_2SO_4 solution.

---

* The salt must be of reagent grade and dried to constant weight before it is used to make up the solution indicated. Distilled water must be free from Na and K. Filter paper must be tested for contamination with Na or K.

† The $CaCl_2$ is prepared from Iceland spar (pure calcium carbonate). To a crystal of Iceland spar in a beaker add 50 ml of exactly 0.1N HCl. Place on a steam bath and leave for several hours until reaction is complete. The solution is then yellow to methyl orange indicator. The entire solution, which should contain 100 mg of Ca as $CaCl_2$, is transferred quantitatively to the volumetric flask which is to be used to measure the total volume of "artificial serum."

Example: To prepare a solution containing K, 0.12 mEq/l, measure 12 ml of stock KCl solution II into a 1000 ml volumetric flask. Add 6 ml of stock Li solution. Other dilutions containing concentrations of K of 0.1, 0.06, 0.05, 0.04, 0.03, 0.02 and 0.01 mEq are prepared in a similar manner, all containing Li in concentrations of 60 mg/l.

LITHIUM SULFATE DILUTE.—It is convenient to prepare a large volume of more dilute Li solution. This should be stored in a large bottle to which a self-filling buret is attached. This serves as a convenient dispenser for the dilution of samples. The stock solution is diluted 1:10 to make a solution of Li concentration 1 g/l. Appropriate volumes can be added to the volumetric flasks into which the samples have been measured.

PROCEDURE FOR DIRECT INTENSITY OR ABSOLUTE METHOD

a) Construction of calibration curves.—SODIUM.—With the flame burning properly and the correct filter in place, the galvanometer is set at zero with distilled water. The standard solution, 1.00 mEq/l, is then introduced and the amplifiers adjusted to give full scale deflection or 100 scale divisions. Distilled water is again introduced and the zero reading checked. If necessary, it is reset. The 100 mark is then reset. These 2 readings are checked several times. Unless the zero and 100 points are maintained on successive readings, gas pressure, air pressure, or both, must be adjusted to give a stable reaction. After proper adjustments so that the curve is relatively constant, the other standards are introduced successively and the readings estimated to the nearest quarter division. From the 5 points (zero, $^1/_4$, $^1/_2$, $^3/_4$ and full scale) thus defined, the curve is constructed. This series should be re-read many times so that any gross variation which occurs subsequently will be recognized. Readings farther from proportionality than 27.5, 52.5 and 77 indicate less than acceptable performance of the instrument.

POTASSIUM.—A similar curve is constructed for K. The 1.25 mEq solution is used to represent full scale deflection. Six points are established: zero, 0.156, 0.313, 0.625, 0.938 and 1.25 mEq. Readings should approximate zero, 14, 27, 52.5, 77 and 100, respectively.

b) Determination of Na and K with 1 Instrument.—SODIUM DETERMINATION.—Before it can be assumed that the instrument is working under optimal conditions, it must be established that the Na of the "artificial serum" can be recovered accurately. One ml portions of this solution are diluted to 500 and 250 ml, respectively, with the distilled water in current use. It is known that the readings should lie between 0.25 and 0.50, and 0.50 and 0.75 mEq, respectively. Therefore the zero point is rechecked, the 0.25 standard is introduced, then the unknown solution diluted 1:500, and finally the 0.50 standard. The 1:250 dilution is then read, followed by the 0.75 standard. Readings are recorded in the following manner:

$$[S_1 \quad S_2]\ U \qquad \text{or} \qquad [S_2 \quad S_1]\ U$$

with S_1 the reading of the standard solution of lower concentration, S_2 the reading of the standard solution of higher concentration, and U the reading of the unknown solution. The 2 points S_1 and S_2 are used to check the calibration curve, on large graph paper (see Fig. 4), and the concentration of the unknown solution is read from the resulting line. This value C represents the concentration of the diluted solution. This is then multiplied by the dilution factor D to determine the concentration in the original material.

$$C \times D = \text{mEq of Na/l}$$

Example: Using "artificial serum" as the unknown (Fig. 4)

	$[S_1$	$S_2]$	U	$C \times D$	$=$ mEq/l	
a)	[25.00	50.50]	27.50	0.275 × 500	= 137.5	av. 138.3 mEq Na
b)	[50.50	75.00]	56.00	0.556 × 250	= 139.0	

a) S_1 = 0.25 mEq standard, S_2 = 0.50 mEq standard D = 1:500

b) S_1 = 0.50 mEq standard, S_2 = 0.75 mEq standard D = 1:250

Only after recoveries have been satisfactory, using "artificial serum," should analysis of the unknown serum be undertaken.

DILUTION OF SERUM.—With pipets calibrated "to contain," 0.20 ml of serum‡ is measured into 50 and 100 ml volumetric flasks. (The flasks should contain a few ml of distilled water. The serum is blown out into the water and the pipet washed by alternately blowing out and withdrawing the diluted serum at least 5 times.) The solutions are then diluted to volume and read in the same manner as the "artificial serum." Examples of readings of various concentrations follow.

Serum	$[S_1$	S_2	U	$C \times D$	$=$ mEq/l	
a)	[25.00	49.50]	48.50	.490 × 250	= 122.5	Av. 123.9 mEq
	[0	25.00]	25.00	.250 × 500	= 125.0	
b)	[26.00	52.00]	29.00	.279 × 500	= 139.5	Av. 139.8 mEq
	[52.25	76.25]	58.00	.560 × 250	= 140.0	
c)	[76.25	51.00]	63.25	.622 × 250	= 155.5	Av. 156.0 mEq
	[51.00	25.25]	31.75	.313 × 500	= 156.5	
d)	[50.00	25.00]	46.75	.467 × 250	= 116.8	Av. 117.2 mEq
	[25.00	0]	23.50	.235 × 500	= 117.2	

---

‡ This is an accurate method of measuring 0.20 ml of serum. However, a solution of lower surface tension, such as the "artificial serum," does not lend itself to measurement with such a pipet. It is important that similar solutions be measured in larger volumes and proportionally diluted (9).

POTASSIUM DETERMINATION.—In Table 1 it will be seen that dilutions of 1:25 and 1:12.5 are recommended. The "artificial serum" is diluted 1:25 ml and 2:25 ml, respectively. (If deflection of the instrument is too slow to yield a maximal reading, the volumes in the latter case should be doubled.) Readings are taken between standards representing 0.156 and 0.313, and 0.313 and 0.625 mEq of K.

Example: Using "artificial serum" as the unknown

	[S_1	S_2]	U	C	×	D	=	mEq/l	
a)	[28.50	55.50]	30.00	0.329	×	12.5	=	4.1	Av. 4.1 mEq
b)	[14.00	29.00]	15.25	0.165	×	25.0	=	4.1	

Since serum is to be diluted with physiologic saline, a blank determination must be made. Rather than read the very small deflection as an absolute value, a 3d dilution of "artificial serum" is prepared and the reading compared with that obtained with the aqueous solution of the same dilution. The difference is of the order of magnitude of $^1/_4$ or $^1/_2$ of a scale division on the galvanometer (0.25 or 0.50). This value is therefore subtracted from the readings obtained on serum before the value is plotted.

Serum samples are then diluted with saline 1:25 ml. The material seldom is sufficiently abundant to permit an additional dilution to 1:12.5. Usually, duplicate dilutions of 1:25 must suffice. However, to verify accuracy of the method, the aforementioned dilutions should be used during the period of preliminary study.

Examples: Typical readings obtained in determinations of serum K follow.

Serum	[S_2	S_1]	U	−	Bl	=	U_1	C	×	D	=	mEq/l	
h)	[15.00	0]	11.50	−	.50	=	11.00	0.115	×	25	=	2.9	Av. 2.9 mEq
	[0	15.00]	11.00	−	.50	=	10.50	0.110	×	25	=	2.8	
k)	[13.00	28.00]	16.00	−	.50	=	15.50	0.180	×	25	=	4.5	Av. 4.6 mEq
	[13.00	28.00]	16.25	−	.50	=	15.75	0.183	×	25	=	4.6	
j)	[14.00	27.75]	14.50	−	.50	=	14.00	0.156	×	25	=	3.9	Av. 4.0 mEq
	[27.75	14.00]	15.00	−	.50	=	14.50	0.163	×	25	=	4.1	
w)	[30.25	15.00]	23.00	−	.50	=	22.50	0.233	×	25	=	5.8	Av. 5.8 mEq
	[13.50	28.00]	20.75	−	.50	=	20.25	0.227	×	25	=	5.7	

PROCEDURE FOR INTERNAL STANDARD INSTRUMENT

The principle is similar to that of the direct intensity or absolute method, differing chiefly in that a fixed amount of Li must be added to both standards and unknown solutions. Lithium is added in the form of a salt, usually the sulfate. Since equal amounts are added to both sample and unknown, it is not essential that the Li solution be exactly quantitative. In practice it is advantageous to observe quantitative precautions so that successive batches of solution may be used interchangeably.

The amount of Li to be added depends on (1) the quality of the filters and (2) the relative sensitivity of the Li cell to the Na or K cell. The proper amount of Li must be determined by experiment for any given instrument. It is measured as that quantity of Li which will give an electrical response equal to that of the concentration of Na or K under consideration (using a particular set of filters and cells) (4). It is customary to choose a Li cell with far less sensitivity than the Na or K cell so that solutions of Li will be 5–10 times as concentrated as the corresponding Na or K solutions (1).

a) *Determination of proper amount of Li to be used.*—In instruments having a direct intensity circuit for Li as well as for Na and K, it is not difficult to determine the proper Li concentration.

(1) Select the standard solution of the highest concentration to be used for the calibration range desired. (For serum Na to be diluted 1:250 and 1:500, a standard which contains 1.0 mEq/l of Na is used.)

(2) Cut in the Na cell only on the direct intensity circuit.

(3) Set the zero reading on the galvanometer with distilled water. By means of the potentiometers set the 1.0 mEq NaCl solution to read 100 divisions.

(4) Cut out the Na cell and cut in the Li cell.

(5) While maintaining the previous settings of the potentiometer, reset zero with distilled water. By trial and error determine the concentration of Li which will give full scale deflection. This will be the optimal concentration to be used.

(6) Repeat, using the K cell and the KCl solution as standard.

In some circumstances it may be advisable to vary from the optimal Li concentration as measured. It will be observed that with a constant Li concentration, readings in the upper part of the dial settings are associated with a steady needle. In the lower part of the dial, where the balance of Li to Na or K is less favorable, an increase in the "noise" or movement of the needle becomes apparent. This condition demands that the null point reading be estimated rather than read., Although an unsteady needle is considered undesirable by many, it does result in greater sensitivity (indicated by a larger difference in the number of divisions on the dial per unit of concentration) which somewhat offsets the disadvantage. Consequently, when Li concentration is increased to bring about greater sensitivity, an unsteady needle must be expected. Sometimes the galvanometer is damped to overcome this unsteady needle and as a result the reading is stabilized. It is unlikely, however, that this procedure results in higher accuracy, even though it appears to be very precise. It may be wise to deviate from the optimal concentration of Li for convenience or uniformity in preparing diluted solutions. If a single dilution of serum, urine, etc., can be used for both Na and K analyses, such a deviation may be very practical.

When the optimal Li concentration has been selected (e.g., 100 mg Li/l for a concentration range of 1.0 mEq Na, or 60 mg Li/l for a range of 0.06 mEq K), the calibration curve is constructed.

b) Construction of calibration curve.—With the flame burning properly and the correct filters in place, the 2 cells to be used (Li and Na, or Li and K) are switched into the circuit. The dial is set at the zero reading and the galvanometer for a center or null point reading. When distilled water is introduced, no deflection of the needle should occur.

The standard of highest concentration in the range previously selected is introduced and the dial reading set at a point slightly below that of maximal reading. The potentiometers are then adjusted to bring the galvanometer reading back to the null point. The dial reading is again set at zero and a Li solution of the selected concentration introduced. The zero and null point readings are again reset. It is important that the Li solution rather than distilled water be used as a zero reading, especially if zero is to be plotted as a point on the curve subsequently.

The standard solutions of the several dilutions selected are then introduced in order successively. This must be repeated several times to be sure that a stable curve results, since slight variations occur in the readings from time to time. Eventually the entire curve may drift a few divisions, so it must be checked repeatedly.

c) Determination of Na and K with 1 instrument.—SODIUM DETERMINATION.—The concentration selected is to read between 0 and 0.75 mEq/l of Na. This requires a concentration of Li of 100 mg/l as determined by the direct intensity method.

The calibration curve is set up using concentrations of 0.125, 0.25, 0.50 and 0.75 mEq/l of Na and 100 mg/l of Li. The zero reading is taken with a solution of 100 mg/l of Li rather than with distilled water. On a dial with 500 divisions, readings are approximately 171, 320 and 485 divisions on the dial for the other 3 points, respectively. The "artificial serum" is diluted 0.5 ml to 250 and 1 ml to 250 to give dilutions of 1:500 and 1:250, respectively. Then 25 ml of the diluted Li solution (1 g/l) is added to each flask.§

---

§ Berliner (3) has demonstrated that it is unnecessary to bring the final volume to an exact dilution. Since the ratio of Na or K to Li is to be read, the final dilution volume can vary as much as 5%. It is, of course, imperative that the measurements of both the Na sample and the Li solution be exact. When large numbers of dilutions are being made, the bubbles at the surface become less of a problem, the need for accurately calibrated flasks is obviated and much time is saved.

Readings are bracketed in the manner described for the direct intensity method.

[S_1	S_2]	U	C	×	D	=	mEq/l	
[156	296]	174	0.278	×	500	=	139.0	Av. 138.2 mEq
[296	456]	233	0.549	×	250	=	137.3	

Serum samples are diluted by measuring exactly 0.2 ml of serum into 50 and 100 ml volumetric flasks. Five and 10 ml of Li solution are added to give a concentration of 100 mg/l. Examples of typical readings follow.

Serum	[S_1	S_2]	U	C	×	D	=	mEq/l	
c)	[175	316]	178	0.256	×	500	=	128.0	Av. 128.3 mEq
	[316	481]	325	0.514	×	250	=	128.5	
d)	[164	314]	187	0.288	×	200	=	144.0	Av. 144.0 mEq
	[323	491]	377	0.576	×	500	=	144.0	
b)	[296	452]	316	0.533	×	250	=	133.3	Av. 132.4 mEq
	[165	294]	172	0.263	×	500	=	131.5	
k)	[298	453]	334	0.557	×	250	=	139.3	Av. 138.9 mEq
	[162	300]	178	0.277	×	500	=	138.5	

POTASSIUM DETERMINATION.—The concentration range selected is between 0 and 0.06 mEq/l. Although it was found by experiment that 60 mg/l of Li was required to balance this concentration of K, it was deemed advisable to use 100 mg/l as in the Na method so that a single dilution would suffice for both analyses. The readings were taken in the same manner as for the Na determination.

Since several concentrations of standard solutions are required, the concentrations of S_1 and S_2 in mEq of K/l are included in parentheses.

Serum	[S_1	S_2]	U	C	×	D	=	mEq/l	
"arti-	[(0.01) 47	(0.02) 106]	85	0.0163	×	250	=	4.07	Av. 4.1 mEq
ficial"	[(0.03) 157	(0.04) 202]	173	0.0335	×	125	=	4.18	
r)	[(0.01) 56	(0.02) 114]	59	0.0105	×	250	=	2.62	Av. 2.6 mEq
	[(0.02) 114	(0.03) 163]	118	0.0208	×	125	=	2.60	
m)	[(0.02) 77	]	77	0.0200	×	250	=	5.00	Av. 5.0 mEq
	[(0.03) 112	(0.04) 153]	151	0.0395	×	125	=	4.93	
e)	[(0.01) 58	(0.02) 111]	89	0.0159	×	250	=	3.97	Av. 3.9 mEq
	[(0.03) 162	(0.04) 208]	164	0.0303	×	125	=	3.79	

NORMAL VALUES

Normal values for serum Na and K, determined by chemical methods, were presented by Hald and Eisenman in 1937 (8). A new series has since been determined using the same chemical techniques and the flame photometer simultaneously (Table 2). In the determination of *sodium*, the differences between measurements by the 2 procedures are not significant. They arise only from the fact that the chemical series included certain subjects with peculiarly low values. In a large series of sera analyzed by both methods simultaneously, differences were consistently within the limits of analytical error. In the case of *potassium*, however, the differences between the measurements by the 2 techniques appear to be significant. The flame photometer technique, as employed in these analyses, is probably the more precise procedure. The chemical method gives wider scattering and especially falsely high values in certain sera.

TABLE 2.—Normal Serum Values for Sodium and Potassium

Method of Analysis	Sex	No. of Observations	No. of Individuals	Max., mEq	Min., mEq	Mean, mEq	S.D.
Sodium							
Chemical and flame photometer	Both*	89	45	144.2	130.5	138.1	±3.14
Potassium							
Flame photometer	M	10	10	4.6	3.6	4.2	±0.33
	F	9	9	4.4	3.7	4.0	±0.28

---

* No significant difference found between males and females.

Sodium and potassium values for about 500 foods and 150 public water samples have been determined by the flame photometer method. Bills *et al.* (5) have presented these values together with incidental comparisons of their method with the chemical techniques on which most of the accepted values are based.

Values for urine and feces are dependent on intake and consequently are extremely variable.

Note.—The author is indebted to many who have contributed to this discussion by personal communications. Since only meager accounts of the biological application of the flame photometer are to be found in the literature, their assistance is gratefully acknowledged. Among those whose gracious generosity should be mentioned are Dr. Robert W. Berliner, Dr. Belton A. Burrows, Dr. Clyde A. Dubbs, Dr. Charles L. Fox, Mr. G. R. Kingsley, Dr. J. V. Petrocelli, Mr. Enoch K. Sprague, Dr. William M. Wallace, Dr. T. E. Weichselbaum and Dr. Van Zandt Williams. No discussion of this instrument would be complete without some mention of the great debt which all of the pioneer users owe to Dr. John W. Berry, whose invaluable assistance and guidance in setting up and maintaining a large number of instruments have been contributed unstintingly. Especial thanks must be given to Dr. John P. Peters for his stimulation and encouragement in the preparation of this contribution.

REFERENCES

1. Barclay Instruments Division, The Patent Button Co., Waterbury 88, Conn.: *Barclay Flame Photometer*, Instruction Manual Model 1BA, 1949

2. Barnes, R. B., *et al.:* Flame photometry: A rapid analytical procedure, Indust. & Engin. Chem. (Anal. Ed.) 17: 605, 1945.
3. Berliner, R. W.: Personal communication.
4. Berry, J. W.; Chappell, D. G., and Barnes, R. B.: Improved method of flame photometry, Indust. & Engin. Chem. (Anal. Ed.) 18: 19, 1946.
5. Bills, C. E., *et al.:* Sodium and potassium in foods and water: Determination by the flame-photometer, J. Am. Dietet. A. 25: 304, 1949.
6. Dubbs, Clyde A., Research Service, Veterans Administration Center, Los Angeles, Calif.: Unpublished data.
7. Hald, P. M.: The flame photometer for measurement of sodium and potassium in biological materials, J. Biol. Chem. 167: 499, 1944.
8. Hald, P. M., and Eisenman, A. M.: The distribution of bases between cells and serum of normal human blood, J. Biol. Chem. 118: 275, 1937.
9. Lavietes, P. H., and Hald, P. M.: Unpublished data.
10. Layton, L.: Flame Photometry, in Annual Reports on the Progress of Chemistry, Section on Analytical Chemistry, London: The Chemical Society 45: 326, 1948.
11. Lowry, O. H., and Hastings, A. B.: Histochemical changes associated with aging: Methods and calculations, J. Biol. Chem. 143: 257, 1942.
12. Lundergardh, H.: *Die quantitative Spektralanalyse der Elemente* (Jena: Gustav Fischer), Vol. I, 1929; Vol. II, 1934.
13. McClelland, J. A. C., and Whalley, H. K.: Lundergardh apparatus: Its construction and its use, J. Soc. Chem. Ind. 60: 288, 1941.
14. National Technical Laboratories, South Pasadena, Calif.: *Beckman Flame Spectrophotometer*, Bulletin 193B.
15. Parks, T. D.; Johnson, H. O., and Lykken, L.: Errors in the use of a model 18 Perkin-Elmer flame photometer for the determination of alkali metals: Interference of common metals, acids and solvents, Anal. Chem. 20: 822, 1948.
16. Perkin-Elmer Corp., Glenbrook, Conn.: *Instruction Manual*, Flame photometer Model 52A, 1949.
17. Stone, G. C. H., and Goldzieher, J. W.: A rapid colorimetric method of the determination of sodium in biological fluids and particularly in serum, J. Biol. Chem. 181: 511, 1949.
18. Wallace, W. M.: Personal communication.
19. Weichselbaum, T. E.: Personal communication.
20. Weichselbaum, T. E., and Varney, P. L.: A new method of flame photometry, Proc. Soc. Exper. Biol. & Med. 71: 570, 1949.
21. Williams, V. Z.: Personal communication.

SECTION III

Studies on Gastrointestinal Pressures, Innervation and Secretions

ASSOCIATE EDITOR, *J. P. Quigley*

INTRODUCTION

THE PRESENTATION of methods of biologic experimentation is readily justified by the fact that experimental results cannot be better than the method by which they were derived. Many modern investigations are essentially a repetition, with supposedly improved methods, of experiments previously conducted. Our successors will undoubtedly repeat some of our experiments with still better techniques than we have used. To a certain extent this situation is inherent in the term "research," which directs us to search and search again. To minimize the wasting of effort and materials in research and the promulgation of conclusions supported by questionable evidence, it is obligatory that we continuously strive to use the best methods available and to improve existing techniques. This critical attitude toward research methods may not have been sufficiently emphasized in studies on the gastrointestinal tract.

Review of investigations on the digestive gives the impression that work in this field lags behind that in many other phases of biology. This cannot be due to lack of important unsolved problems in gastroenterology. Perhaps it reflects a paucity of inspiring research leaders; or a major cause may be a reluctance to apply techniques with appeal to young investigators.

For many years the collection of *general* information regarding the digestive tract, e.g., studies of gastrointestinal motility by the balloon-water manometer technique, was considered adequate. This attitude was justified since better methods were not available. However, the modern investigator can rarely be content with data collected by such uncritical methods. He should attempt to develop procedures which more accurately measure the physical or chemical factors on which the investigation depends and shun the application of such procedures as statistical analysis and planimetric measurements to records obtained by methods which are physically incapable of supplying accurate data.

A research technique is not better solely because it is new or involves complex procedures. Although the day is essentially past when a piece of apparatus constructed from "a strip of wood, some soft iron wire and a rubber band" will provide data sufficiently accurate to meet modern standards, a simple device having an adequate "figure of merit" will be preferred to a more complex one. Jacques Loeb said that any fool can devise a complex experiment but it requires a genius to devise a simple one.

A number of techniques, such as those using electronic equipment and radioisotopes, have become available which provide potential methods of investigation. Gastroenterologists, like other biologists, depend largely on fundamental methods developed by others, chiefly physicists and chemists. It is the responsibility of the investigator who adapts such techniques to his studies to certify that their use constitutes a real advance and not simply a substitution of a questionable new method for a questionable old one. To insure accuracy in studies employing complex electronic equipment or tracer techniques, the investigator does well to form a close alliance with a physicist or electronic engineer who can provide the essential critical attitude. Without such a precaution, too many investigators collect figures but not authentic data.

In gastrointestinal research it is probably most obligatory that physiologic experiments be conducted under the most physiologic conditions attainable. Significant studies on the normal movements and secretory activities of the digestive tract usually cannot be made on animals under a general anesthetic. Serious training of the unanesthetized subject, human or animal, is a prerequisite to a study by any of the techniques in common use. This point is emphasized because of numerous reports of observations on subjects, especially human subjects, who have not gone through a suitable preliminary training period.

Most investigators can profitably devote a greater fraction of their research time to a more careful designing of their projected studies. The actual experiments will then be likely to progress more rapidly, more smoothly and to have greater significance. It may not be amiss to extend a word of caution regarding the premature reporting of experimental results. We are less likely to be embarrassed later if we permit our reports to stand briefly "so the gas can escape" than if we rush them in a half-baked condition into publication. Carefully designed investigation conducted under the most optimal conditions with physically adequate equipment and critical evaluation of the results distinguishes classic studies from those in the pot-boiler category.

—J. P. Quigley

Registration of Digestive Tract Intralumen Pressures*

DANIEL A. BRODY *and* J. P. QUIGLEY, *University of Tennessee*

In 1895 Frank (5) formulated the theoretical principles and mathematical equations for construction of an accurate pressure measuring device and Wiggers (14) in 1928 stated the requirements for an accurate manometer. These principles are applied in modern cardiovascular research and meticulous efforts are made to record pressures accurately. The value of investigations in this field has been enhanced by the resulting fidelity. Investigators of gastrointestinal motility have almost universally ignored these facts and have not insisted on exactness in intralumen pressure concepts.

Some gastroenterologists have referred in their reports to "pressure," although pressure actually was not the type of gut activity studied. Some have indicated that their apparatus accurately recorded the intralumen pressure but presented no confirmatory evidence. The basic principles of the apparatus used in these studies usually prevented accurate registration of pressure. Some gastroenterologists have introduced unprofitable complexities into their apparatus and others have introduced modifications which ignored physical laws. Others, because they continued to use inaccurate techniques for pressure measurement, probably have failed to enhance the value of their data by planimetric or statistical analysis of their fundamentally questionable records.

Since intralumen pressures of the gut *can* be accurately measured and accurately expressed, we urge that the term "pressure" be rigorously reserved for the results of studies involving a serious effort at accuracy. General terms such as "motility" and "motor activity" can be used with appropriate qualifying adjectives to express the results from studies with such apparatus as the balloon and water manometer when the investigator does not desire to emphasize accuracy in pressure measurement.

The methods employed for the registration of intralumen pressure of the digestive tract before 1938 were undependable for accurate results since they incorporated 1 or more of the following objectionable physical characteristics: (*a*) a naked balloon which on constriction by the gut wall produced intraballoon pressures not accurately reproducing the intralumen pressures, (*b*) an apparatus having so large a volume/pressure coefficient that a complex was recorded of pressure and volume changes, (*c*) a recorder too sluggish in dynamic response (inertia too great) to permit faithful registration.

---

* Aided in part by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

We have critically analyzed the basic requirements for pressure registration and, through application of the principles which developed, have accurately and continuously recorded intralumen pressures from the digestive tract by (*a*) the pressure conduction method and (*b*) the in vivo transducer method (11). This subject is still in a state of flux and several modifications of these 2 primary principles have been, or are being, developed in various laboratories in a search for the best type of equipment. However, a suitable device for use in the laboratory or at the patient's bedside can now be assembled from commercially available equipment or easily constructed parts.

FUNDAMENTAL PHYSICAL CONSIDERATIONS

Errors inherent in naked balloon.—Those pressures in the lumen of a gut segment which arise from contraction of the gut musculature on fluid or semifluid contents may be expressed by the formula

$$P = (dV/dt)\cdot R \qquad (1)$$

where P is the intralumen pressure at any instant, dV/dt is the rate at which material escapes from the gut segment, and R represents the total resistance to the escape of contents. When the pressures of the gut are being measured, R may be further expressed as

$$1/R = 1/R_p + 1/R_d + 1/R_m \qquad (2)$$

where R_p, R_d and R_m represent, respectively, the resistance offered to an escape of material in the proximal direction, the distal direction and into whatever manometric system has been introduced for the registration of pressure. Unless the magnitude of R_m is high, it obviously will seriously modify R and, in turn, significantly alter P, which represents the pressure in the gut cavity.

If the pressure-sensing device is a naked balloon lying within the lumen of the gut, it constitutes a part or all of the contents of the segment. The balloon then creates an artificial barrier to the escape of contents which will usually differ from the natural barrier. Thus, the R_p and R_d, and consequently R, of equation (2) will be altered in an unpredictable and inconsistent manner. Accordingly, the magnitude of pressures recorded from the naked intralumen balloon bears no consistent relationship to true intralumen pressures. (A more rigorous analysis of this subject has been reported (10).)

Volume/pressure coefficient of recording device.—Schreiber (12) attempted to overcome many of the fallacies resulting from use of the unprotected intralumen balloon by recording from a simple open-ended tube placed in the gut. However, the effects of this excellent innovation were vitiated by connecting the tube to a U-tube water manometer which possessed a large volume/pressure coefficient and provided a significant artificial channel for the escape of gut contents. Thus the pressure developed in the gut cavity in which the tube tip was located was distinctly modified. For accurate registration of intralumen pressures a manometer possessing a small volume/pressure coefficient should be chosen to provide a value of R_m sufficiently great to permit only minimal distortion of R (cf. equation (2)).

Other advantages result from use of pressure recording devices with very low volume/pressure coefficients (1). (*a*) There is always enough material in the gut to "drive" such a manometer even during the fasting state. Conversely, the quantity of material in the gut during fasting may only permit the registration of the lower portion of a pressure change by a high volume/pressure device. (*b*) When the size of the gut cavity is not reduced by escape of contents through the recording tube, its volume can alter in a natural manner rather than follow an artificial pattern. This can be significant, since the efficiency of a hollow organ in creating intralumen pressure is greatly influenced by the volume of its cavity (9). Thus the pressure created in a gut section from which the contents escape only through natural channels may differ considerably from that occurring when some of the contents also escape into a high volume/pressure device.

Dynamic requirements of pressure-recording device.—Since gut movements are relatively slow, it might be assumed that intralumen pressures may be accurately recorded with rather sluggish devices. However, with adequate apparatus having little inertia, we have shown the occurrence of surprisingly rapid pressure changes in intralumen pressures. We recorded (10) pressure waves which rise in an essentially linear manner from the basal to the maximal pressure in approximately 1 sec and decay in a similar manner to the basal pressure during the subsequent 1 sec interval. This appears to be the type of intralumen pressure wave which is the most exacting to be recorded from the dynamic standpoint. By idealizing this pressure spike into an isosceles triangle which has a time base of 2 sec, one may calculate the response of a pressure recorder from solution of the following fundamental equation of motion

$$M \frac{d^2s}{dt^2} + 2k\sqrt{EM} \cdot ds/dt + Es = Qf(t) \qquad (3)$$

M represents the mass of the pressure-sensitive element in the recorder, E its modulus of elasticity, and s its displacement from the resting position, k = damping coefficient, t = time, Q = pressure-sensitive area and $f(t)$ represents pressure as a time variable function. All values are expressed in cgs units. The exact expressions for $f(t)$ will be

$$\text{Ascending limb:} \quad f(t) = P_{\max} t \tag{4}$$

$$\text{Descending limb:} \quad f(t) = P_{\max}(1 - t) \tag{5}$$

$$\text{Recovery phase:} \quad f(t) = 0 \tag{6}$$

where $P_{\max}$ = peak pressure.

The physical constants of the recording device will appear in the solutions of equation (3). The inadequacies of the conventional liquid-filled U-tube manometer are demonstrated by our calculation of the response of such a manometer which is critically damped ($k = 1$) and in which the ratio $E/M = 100$. Such a manometer may record a maximal pressure of approximately 19.6 cm H_2O when filled with water; 46.6 cm H_2O when filled with Bromoform and 266 cm H_2O when filled with Hg.

TABLE 1.—CALCULATIONS FROM EQUATIONS OF MOTION

TIME, SEC	L—IDEAL	L—ACTUAL
0	0	0
Ascending limb		
0.2	0.200	0.054
0.4	0.400	0.211
0.6	0.600	0.402
0.8	0.800	0.600
1.0	1.000	0.800
Descending limb		
1.2	0.800	0.892
1.4	0.600	0.778
1.6	0.400	0.596
1.8	0.200	0.399
2.0	0	0.200
Recovery phase		
2.2	0	0.054
2.4	0	0.011
2.6	0	0.002
2.8	0	0.000

The derived equations of motion are

$$\text{Ascending limb:} \quad L = \left(\frac{2}{\sqrt{E/M}} + t\right)e^{-\sqrt{E/M}\,t} + t - \frac{2}{\sqrt{E/M}} \tag{7}$$

Descending limb: $$L = \left(\frac{2}{\sqrt{E/M}} + t\right)e^{-\sqrt{E/M}\,t} - t + 1 + \frac{2}{\sqrt{E/M}} \tag{8}$$

Recovery phase: $$L = \left(\frac{2}{\sqrt{E/M}} + t\right)e^{-\sqrt{E/M}\,t} \tag{9}$$

where L is the ratio of actual response at any instant to the *ideal* peak response and e is the natural logarithmic base. Calculations from this set of equations, given in Table 1 and presented graphically in Fig. 1, show significant departures from an ideal response.

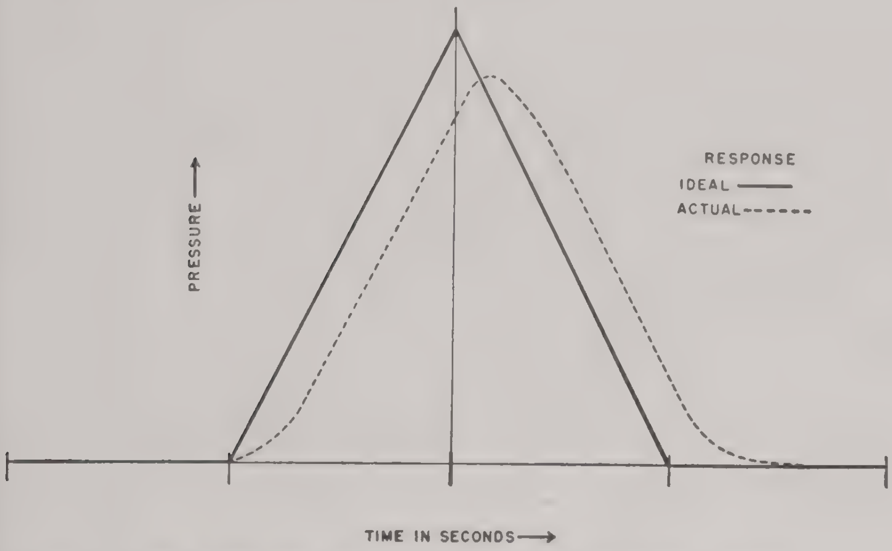

FIG. 1.—Ideal response curve compared with distorted response of dynamically inadequate pressure recorder (U-tube manometer) to rapid intralumen pressure changes. (Discussion in text.)

A simplified method of calculating the fidelity of response derives from further analysis of the fundamental equations. For manometers with a damping coefficient, $0.65 \leq k = 1$, and a natural undamped frequency (f_0), $f_0 \geq 2$ c/sec, it may be shown that the percent deviation, Δ, of the actual response from the ideal response at the instant of peak pressure will be

$$\Delta = 100\,\frac{k}{\pi f_0} \tag{10}$$

Therefore, to achieve with a critically damped apparatus an error no greater than 1% at the instant of peak pressure, calculation from equation (10) shows that the minimal natural undamped frequency of the equipment would be 31.8 c/sec.

PRINCIPLES OF PRESSURE CONDUCTION METHOD

The principles of this technique are exemplified in Figures 2 and 4. The component parts consist of an open-ended cylindrical tip, a small-bore connecting tube and a pressure-sensitive recorder. The connecting tube and tip contain a gas, usually air. If the volume of the pressure recorder is very small, it may contain the same gas (Fig. 2). However, if this volume is comparatively large, it must be filled with a relatively incompressible fluid such as water (Fig. 4). Pressures in the gut cavity tend to force the fluid or semifluid contents into the open end of the tip. This ingress of material is resisted by the elastic recoil of the gas volume of the apparatus and of the pressure-sensitive device. At the same time the intralumen pressures are communicated through the tip and connecting tube to the pressure recorder, thus producing tracings of intralumen pressures.

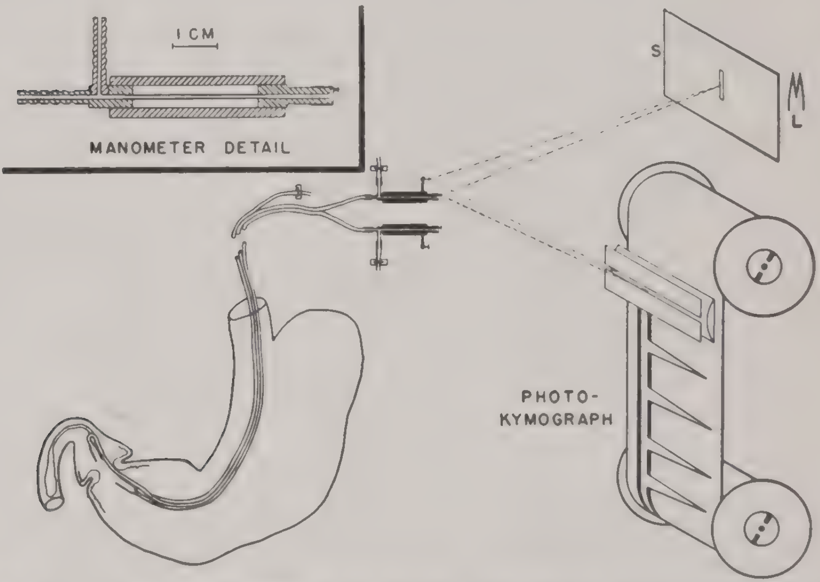

Fig. 2.—Two-channel open tube optical manometer technique. *L*, projection lamp, having slit, *S*. For further details see Figure 3 and text. (From Brody and Quigley (2), with permission.)

These 3 components possess individual inherent dynamic characteristics, and the over-all quality of response tends to be limited by that of the portion possessing the poorest dynamic characteristics. In this respect the pressure recorder is usually decidedly superior to the tip or connecting tube; therefore, one of these latter components determines the over-all dynamic adequacy of the device. We have not succeeded in developing the exact equations of tip and connecting tube dynamics, but we have derived approximate solutions which are of great value in designing the apparatus and understanding its function.

Dynamics of the connecting tube.—A pressure impulse from the tip will be conducted through the connecting tube with the velocity that sound travels through the tube. If the pressure to which the tip is exposed is varying periodically, the contents of the connecting tube will be in resonance with those components which possess a frequency of

$$f = n\,C'/4L$$

where f = frequency, C' = velocity of sound in the tube, L = the length of the tube, and n = any odd integer.

Many factors modify the velocity of pressure transmission in the connecting tube. Pressure pulses of large amplitude are transmitted more rapidly than those of small amplitude. Transmission proceeds more slowly through small-bore than through large-bore tubing. High frequency pressure pulses are transmitted more rapidly than those of low frequency (15).

Transmission characteristics may be favorably influenced by selecting the proper type of gas for the conducting medium (11). For instance, the velocity of pressure pulses is almost twice as great in helium as in air; in hydrogen it is twice as great as in helium. For very small lumen tubing, helium is only slightly better than air because its viscosity is slightly greater than that of air. However, hydrogen with its low viscosity is decidedly superior to either air or helium.

Dynamics of the tip.—We determined (1) that if the gas volume in the manometric system is regarded simply as an elastic cushion against which the intralumen pressure forces gastrointestinal contents, an extension of the Frank (6) equation leads to the relationship

$$f_0 = \frac{1}{2\pi l}\sqrt{\frac{\gamma P_0}{\mu S}\cdot\frac{Vt}{V_0}}$$

where f_0 = natural, undamped frequency of the tip, l = length of tip, γ = specific heat of the gas, S = specific gravity of gastrointestinal contents, P_0 = atmospheric pressure, Vt = volume of tip and V_0 = total gas volume of the device at atmospheric pressure, μ is an arbitrary constant such that the movements of a "plug" of liquid having an area equal to the internal area of the tip and a length μl are dynamically equivalent to the vibratory motion of the gastrointestinal contents.

General considerations in design of apparatus.—Gas, rather than liquid, is preferred as the pressure-conducting medium because (*a*) it permits use of smaller and more flexible, i.e., more "physiologic" tubing than with liquid conduction, and (*b*) it is not necessary to know the vertical distance between tip and manometer, i.e., no correction is required for hydrostatic pressure of fluid in the tube. However, total gas volume must be as small as possible to make the volume/pressure coefficient minimal.

A number of conflicting factors enter into design of the tip. From the purely dynamic standpoint it is desirable to make the tip short and very wide. However, it must be narrow enough for the gastrointestinal contents to form a stable meniscus in it. The smaller the tip volume the more favorable the volume/pressure coefficient, but tip volume must be large enough so the maximal anticipated intralumen pressure will not force gastrointestinal contents into the connecting tube and produce plugging. Since the tip constitutes a foreign body, it is desirable physiologically to have the tip small with an external length approximately twice the external diameter. Despite such conflicts in theory, it has been possible to construct tips which satisfactorily meet these various requirements.

The connecting tube should be of minimal length and minimal bore; however, the bore must not be so small that it causes excessive damping. The compressible volume of the associated manometer and of any T-tubes should also be small.

Hansen (7) demonstrated that transmission of periodic pressure variations through liquid-filled, flexible catheters is closely analogous to transmission of electrical signals along telephone lines. Application of this fundamental electrical theory to our pressure conduction technique should result in a more exact formulation of its dynamic characteristics.

2-CHANNEL OPTICAL MANOMETER METHOD

The apparatus utilizing the pressure conduction technique most extensively studied by us is the optical manometer (2). We usually employ it in a 2-channel form so prepared as to record in human subjects simultaneously from 2 regions approximately 25 mm between the recording tips.

a) Tip construction.—Details and manner in which the tips are attached to the connecting tubes are illustrated in Figure 3. Each tip consists of 11 mm of Koroseal tubing of 3.65 mm internal and 5.10 mm external diameter (nominal bore, $^1/_8$ in.). The distal end of each tip is reinforced by inset of a brass ring 0.25 mm thick, 2.5 mm long and 3.65 mm internal diameter. The proximal end of each tip is fitted to a brass reducing connector with $^1/_{16}$ in. bore. A 5 cm length of no. 16 tubing† is forced over the small end of each connector. The hairpin turn in the more distal tubing is made by slipping a clean malleable wire of the same diameter as the bore into a length of tubing. Then with alternate bending and cautious flaming at the bend, a sharp 180° turn is made. If flaming has been properly performed the wire may now be removed and the tubing will retain the sharp U-bend.

b) Connecting tubes.—To make the tubes which connect the tips with the optical manometer, no. 20 Irvolite Transflex tubing† is slowly stretched at 100 C to about half again its original length and permitted to cool, decreasing the original bore to about 0.75 mm diameter.‡ Of the modified tubing, 100 cm comprises the main portion of each connecting tube. One end is sealed into the open end of the no. 16 tubing; the other end is connected to a 5 cm length of 1 mm bore, thick-walled rubber tubing which facilitates connection to the optical manometer and also can be pinched closed without damage to the tubing.

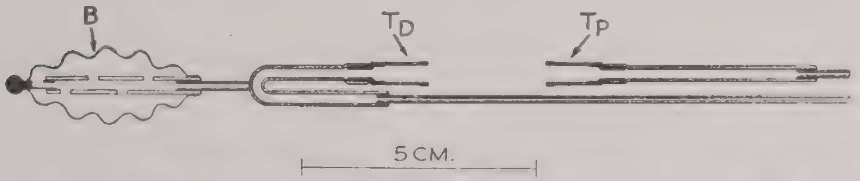

FIG. 3.—Detail of open-end tip construction for 2-channel open tube optical manometer intralumen pressure recorder (cf. Fig. 2). *B*, balloon; T_D, distal open-end tip; T_P, proximal open-end tip. Only the distal portion of the tube to the balloon is shown. (Discussion in text.)

c) Optical manometer (Fig. 2).—All parts are constructed of brass except a short length of 23 gauge stainless steel tubing which joins the proximal and distal parts of the manometer. The joints involving the stainless steel tubing are made first with silver solder; remaining joints are made with soft solder. The membrane is rubber sheeting, usually 0.016 in.§ tied in place while stretched to 4 times its original dimensions. The mirror consists of a 2 mm square chip of a thin 0.5 diopter plano-convex lens which has been silvered on the plane surface. The mirror is cemented to the appropriate edge of the membrane on a hard rubber peg 1 mm in diameter and 1 mm long. Soft rubber tubing of 1 mm bore is attached to the side arm and sealed on with soft laboratory wax.

d) Assembly.—The 2 pressure-conducting tubes and tips and a 3d tube are bonded into an integral assembly by lashing them together at appropriate places with a wrapping of fine silk thread which is then coated with Glyptal lacquer. The 3d tube extends 5–6 cm beyond the distal pressure-conducting tube and terminates in a small balloon partially filled with Hg to assist intubation of the small intestine. After the recording tip has reached the portion of the gut from which pressure is to be measured, the Hg is aspirated. A 50 cm length of $^1/_4$ in Penrose drainage tubing covers the portion of the 3 tubes above the proximal recording tip.

---

† Obtained from the Irvington Varnish and Insulator Co., Irvington, N. J.

‡ No. 24 tubing, with 0.7 mm lumen is now available from Irvington Varnish and Insulator Co., Irvington, N. J.

§ Supplied by Anode Rubber Company, Akron, O.

e) Introduction and application.—Before the tubing assembly is introduced into a subject, lightly stretched condom rubber membranes are tied with fine thread over the open distal ends of the recording tips. The oropharynx is anesthetized with a spray of 2% butacaine sulfate and the upper esophagus with 2 or 3 cc of the same solution injected through a short tube introduced into this region. The subject seldom has difficulty in swallowing the assembly. After the distal end is well into the stomach, the proximal ends of the tubes are fastened to the distal end of a soft rubber catheter which has been passed through a nostril into the oropharynx and out the mouth. This catheter is then retracted so the final route of the tubing assembly is pernasal. The tips are observed fluoroscopically until they have been maneuvered into the desired location. Administration of a small quantity of thin barium sulfate suspension increases the accuracy of localization, especially when the pyloric sphincter region is being studied.

When the tips are in position, 3–5 cc of air is forcibly injected into each of the connecting tubes, which are then clamped off while the air is flowing. This procedure cleanly ruptures the protective condom rubber membranes away from the open ends of the tips. The connecting tubes are then joined to the optical manometers. Following release of the clamps on the connecting tubes, the manometers record intralumen pressure. After pressure recording has begun, extreme care must be taken at all times to avoid leaks in the manometric system. Otherwise, gastrointestinal contents tend to enter and plug the fine-bore tubing. Despite extensive precautions, air tends to leak slowly from the system. The routine forcible injection of 2–3 cc of air through the T-tube every 15 min compensates for this tendency and also expels any accumulated gastrointestinal contents from the tip. Plugging then is infrequent, is readily recognized and is usually released by forcible introduction of air.

f) Evaluation.—Investigations of the physical characteristics of this device and results of 187 runs on 36 human subjects indicate that it is rather satisfactory. The total internal volume of the manometric system is 0.8 cc, with tip volume 0.1 cc. This permits the recording of pressures up to 150 cm H_2O maximum before gastric contents begin to enter the fine tube. The volume/pressure coefficient is 0.08 cc/100 cm H_2O. The natural frequency under conditions of use is approximately 35/sec, with damping coefficient 0.5–1.0 depending on the nature of the gastrointestinal contents. The computed accuracy with which peak pressures of the most rapid type are recorded (equation (10)) is better than 99%.

OPEN TUBE ELECTROMANOMETER TECHNIQUE

This is essentially an extension of the open tube optical manometer technique, with an electrical pressure transducer (Sanborn Electromanometer) substituted for the optical manometer (11).

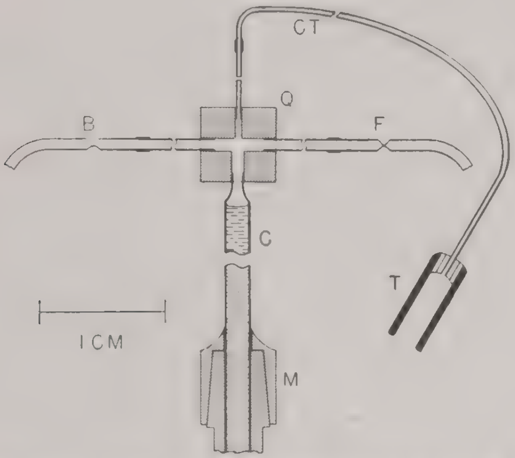

FIG. 4.—Application of low volume/pressure transducer (Sanborn Electromanometer) to registration of intralumen pressures. *T*, open-end tip; *CT*, fine-bore connecting tube; *Q*, 4-way tubing connector; *F*, side-tube used for flushing; *B*, "bleeder"; *C*, glass capillary containing liquid-gas interface; *M*, fitting to electromanometer. (Discussion in text.)

Details of the apparatus are shown in Figure 4. Other types of low volume/pressure coefficient transducers such as the Statham strain gauge and Clark capsule apparently give equal success.

A small, relatively soft plastic tip 3 mm in external diameter and 8 mm in over-all length lies in the lumen of the gut. Intralumen pressures received by the open end of this tip are transmitted by the gas column in the tip and the 0.4 mm bore flexible plastic tubing to a gas-water interface in the glass capillary tube passing through a 4-way tube which also has connections to a source of helium or hydrogen and to the atmospheric air. From this interface, further transmission of pressure proceeds through water to the electromanometer which converts intralumen pressures into proportional electrical signals which may then be recorded by an optical galvanometer or suitable direct-writing apparatus calibrated in terms of intralumen pressure.

Our application of this method is limited to the gastroduodenal region of well trained, unanesthetized dogs provided with gastric and duodenal cannulas according to the technic of Meschan and Quigley (9). During operation of the device the connection of the 4-way tube to the atmosphere is sealed off. The other outlet tube has a very fine constriction (the "bleeder," *B*, Fig. 4) which permits clean, dry gas under pressure to leak steadily into the system at the rate of 3–5 cc/hr. This arrangement minimizes greatly the possibility of plugging. Theoretical considerations and extensive trials have demonstrated that this introduction of gas does not perceptibly distort the pressure tracings.

Use of air in the system is fairly satisfactory from the dynamic standpoint. Helium is somewhat better than air and hydrogen is superior to either. Hydrogen induces an adequate dynamic response from tubing lengths up to 100 cm, which are sufficient for many applications in the intact human subject.

When 40 cm of fine-bore connecting tubing is used, total gas volume of the system is approximately 0.1 cc. The volume/pressure coefficient under these conditions is 0.01 cc/100 cm of H_2O. Since tip volume is 0.04 cc, the apparatus is capable of recording about 660 cm H_2O pressure before gastrointestinal contents begin to enter the narrow tubing and cause plugging. The maximal pressure we have so far recorded from the pyloric antrum is 475 cm of H_2O. The over-all dynamic response of the equipment is approximately the same as that of the open tube optical manometer arrangement.

Critique.—The present form of apparatus represents a satisfactory compromise of the physical and physiologic factors involved which are sometimes antagonistic to each other and sometimes concordant.

Gas, rather than liquid, is used as the pressure-conducting medium in the tubing assembly since it obviates correction for the hydrostatic effect of fluid in the tubing. The low viscosity of gas also permits use of exceptionally fine-bore connecting tubing. Certain errors occur because of the pressures developed at the open end of the tip by capillary attraction and bubble formation. These pressures are usually small and can be corrected if a very accurate determination of basal pressure is desired.

Although use of the open-ended tip eliminates most of the errors which arise from presence of an unprotected balloon in the gut, it still introduces certain artificial conditions which might interfere with accurate registration of peak phasic pressures. As the gut contracts firmly on the tip during development of peak pressures, the tip props the gut open and interferes with the final phase of contraction. We do not know how large an error arises from this interference, but we have evidence that size of the tip may be significant. With the present apparatus, having an external tip diameter of 3 mm, we have frequently recorded peak pressures of 300 cm of H_2O from the distal antrum. Previously used apparatus, having a tip diameter of 5 mm, rarely recorded pressures in excess of 90 cm of H_2O from the same region. A plateau does not form in the recorded phasic pressure spikes. This indicates that the volume/pressure coefficient is so small that even during maximal contraction enough gut contents are present to "drive" the manometer.

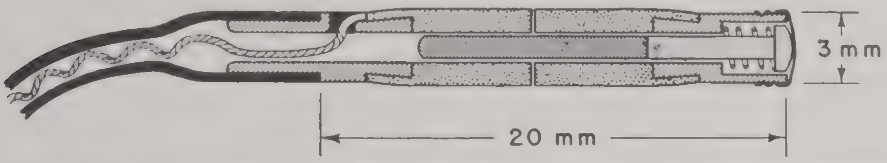

FIG. 5.—Miniature in vivo transducer used to record intralumen pressures of gut (3). The body consists of lacquer-impregnated center tapped coil (coarse stippling) with brass head and tail stocks (coarse cross-hatching). Plunger in the coil is made of aluminum alloy (fine stippling) with attached soft iron slug (fine cross-hatching). Plunger is elastically mounted by a fine helical spring in the head stock and a rubber membrane tied across the head stock. The transducer comprises 2 arms of a balanced induction bridge. Changes in gut intralumen pressure cause proportional displacements of plunger. Resulting unbalance potentials are amplified and recorded as intralumen pressures which displaced plunger.

The dynamic characteristics of our apparatus have been determined through analysis of square-wave responses. At the kymograph speeds of 7.5 cm/min which we ordinarily use, the square-wave response closely approximates the ideal. The transient pressure changes which occur in the gut are less rapid than the square-waves. Thus the apparatus apparently faithfully records intralumen pressures from the gut.

APPLICATION OF IN VIVO TRANSDUCERS TO REGISTRATION OF INTRALUMEN PRESSURES

The application of miniature electrical transducers small enough to be placed in the gut lumen is still in an early stage, but it is anticipated that apparatus of this type will be developed that is sufficiently reliable, compact and durable to encourage its use at the bedside and in the clinic as well as in the laboratory.

Miniature transducers based on the principle of the carbon

TABLE 2.—Comparison of Methods for Recording Intralumen Pressures of Organs

Characteristics	Open Tube Optical Manometer	Open Tube Electromanometer	In Vivo Transducer
Stability	Excellent	Fairly good	Apparently satisfactory
Adjustment of sensitivity	Difficult; impossible during experiment	Easy, even during experiment	Easy, even during experiment
Check of baseline & calibration	Possible during experiment	Possible during experiment	Impossible during experiment
Freedom from plugging	Fair	Good	Excellent
Vol/pressure coefficient	Fair	Good	Excellent
Feasible to record very large pressure (over $^2/_3$ atm)	No	Yes	Yes
Technical difficulties	Mod., especially membrane & mirror mounting	Slight	Moderately great
Feasible to use direct writer	No	Yes	Yes
Mobility, compactness	Poor, rather cumbersome	Fair when used with direct writer	Potentially good
Freedom of movement of subject	Poor	Poor; probably fair with Lilly or Hathaway type transducer	Good
Commercial availability	All parts but tubing assembly	All parts but tubing assembly	Transducer (from Gauer); recording apparatus
Cost	Low, espec. with multiple channel recording	Components moderately expensive	High; major components custom made

button microphone, the variable electrolytic cell and the variable capacitor have not proved satisfactory in our hands. We developed an electromagnetic transducer in which an elastically mounted iron slug, moving in response to pressure variations, caused changes in self induction (Fig. 5). This device has yielded encouraging results (11). Code (3) has recorded intralumen pressure with Gauer's (4) modification of the miniature differential transformer of Wetterer (13).

The electromagnetic devices employed by ourselves and by Code are driven at audiofrequencies (6000 c/sec). Keisker (8), working in our laboratory, has succeeded in producing prototypes of electromagnetic transducers which may be driven at radio frequencies of at least 100 kc/sec. An important factor in Keisker's success is his ingenious development of an extremely fine, shielded lead consisting of 3 lengths of no. 40 copper wire twisted together in a slow spiral. One wire is Formex insulated and the other 2 are bare. Coating this assembly with silver paint, such as is used in "printed" circuits, provided excellent electrical shielding which is reasonably durable. The inherently greater sensitivity of radiofrequency devices should permit smaller transducers with more durable membranes than are possible with audiofrequency devices.

REFERENCES

1. Brody, D. A., and Quigley, J. P.: Unpublished observations.
2. Brody, D. A., and Quigley, J. P.: Intralumen pressures of the stomach and duodenum in health and disease, Gastroenterology 9: 570, 1947.
3. Code, C. F.: Personal communication.
4. Ellis, E. J.; Gauer, O., and Wood, E. H.: Application of a manometric sound to the recording of intracardiac and intravascular pressures, Proc. Staff Meet., Mayo Clin. 25: 49, 1950.
5. Frank, O.: Zur Dynamik des Herzmuskel, Ztschr. f. Biol. 32: 370, 1895.
6. Frank, O.: Kritik der elastischen Manometer, Ztschr. f. Biol. 44: 445, 1903.
7. Hansen, A. T.: Pressure measurement in the human organism, Acta physiol. Scandinav., Vol. 19, supp. 68, 1949.
8. Keisker, H. W.: Unpublished observations.
9. Meschan, I., and Quigley, J. P.: Spontaneous motility of the pyloric sphincter and adjacent regions of the gut in the unanesthetized dog, Am. J. Physiol. 121: 350, 1938.
10. Quigley, J. P., and Brody, D. A.: Digestive Tract: Intralumen Pressures: Gastro-Intestinal Propulsion, Gastric Evacuation, Pressure-Wall Tension Relationships, in Glasser, O. (ed.): *Medical Physics* (Chicago: Year Book Publishers, Inc., 1950), Vol. II.
11. Quigley, J. P., *et al.*: Accurate registration of intralumen pressures of the digestive tract by two new methods, Federation Proc. 9: 102, 1950.
12. Schreiber, J.: Zur physikalischen Untersuchung des Oesophagus und des Magens (mit besonderer Berücksichtgung des intrathoracalen und intra-abdominalen Drucks, Deutsch. Arch. f. klin. Med. 33: 425, 1883.
13. Wetterer, E.: Eine neue manometrische Sonde mit elektrischer Transmission, Ztschr. f. Biol. 101: 332, 1943.
14. Wiggers, C. J.: *Pressure Pulses in the Cardiovascular System* (London: Longmans, Green & Co., Ltd., 1928).
15. Wood, A. B.: *A Textbook of Sound* (London: G. Bell & Sons, Ltd., 1932), pp. 238 f. and 263–65.

Preparation of Esophageal Fistulas and Gastric Pouches

M. H. F. FRIEDMAN, *Jefferson Medical College*

The first gastric fistula, established specially for the purpose of experimentation, was made by Bassov (4) of Russia in 1842 and practically simultaneously by Blandlot (7) of France in 1843 (16). Each investigator independently attempted to reproduce in the dog the accidental preparation in Alexis St. Martin which Beaumont (5) had described 10 years earlier. For some physiologic studies the gastrostomy preparation was a distinct advantage over the older method of intubating the stomach per os, but for studies of gastric secretion it was unsatisfactory because it did not permit quantitative collection of gastric secretions or insure against contamination of the juice with saliva, esophageal secretion and regurgitated duodenal contents.

In 1889 Pavlov and Simanovskaia (31) performed an esophagostomy on a dog with a gastric fistula and could obtain relatively pure gastric juice of high peptic activity by sham-feeding the dog. This did not prevent contamination of gastric contents by regurgitated duodenal contents and failed to provide a means of studying simultaneously the mechanism of gastric secretion and the processes of gastric digestion. Nevertheless, the esophagostomy operation has remained an important tool especially for investigating the nervous control of various gastrointestinal secretory and motor problems.

In 1875 Klemensieviciz (23) attempted to adapt to the pyloric part of the stomach the principle of the Thiry loop of the intestine, but the dog lived only 3 days. In 1878 Heidenhain (18) reported successful preparation of a pouch from the pyloric part of the stomach and in 1879 or 1880 of a similar pouch from the fundus. Frémont (14) in 1895 applied the Thiry principle to the whole stomach; the esophagus was anastomosed directly to the small intestine and the entire stomach, with cardiac and pyloric orifices closed, drained through a gastric cannula to the exterior.

Both the Frémont and Heidenhain pouches were useful for obtaining pure gastric juice and are still extensively used. The operative procedure in each instance deprives the pouch of most or all of its vagal innervation. The Heidenhain pouch is usually deprived of all vagal fibers but retains its sympathetic nerve supply which reaches the stomach along the vascular supply. For study of purely humoral mechanisms of gastric secretion a completely denervated type of pouch was devised by several investigators. These include the Bickel pouch of the stomach and various portions of the stomach transplanted as pouches to subcutaneous areas.

Pavlov developed a pouch of the fundus which he believed retained most of its vagal nerve supply. The principle is based on the fact that the vagal fibers run over the gastric muscularis superficial to the mucosa and penetrate toward the mucosa only at a point close to their termination in the submucous plexus. Cutting through the mucosa alone thus essentially leaves the vagal fibers intact. The pouch designed by Pavlov and Chigins (30) and used widely later is now known to be only partially innervated by vagus fibers. Since the posterior vagus trunk does not pass along the greater curvature of the stomach, as earlier investigators believed, most of the vagus fibers to the pouch are severed when the classic Pavlov procedure is used. In recent years operations have been devised for making pouches which retain intact most of the vagal fibers. Since the term "Pavlov pouch" has come to be used synonymously with "intact vagal innervation," some of these later types of pouches, notably that made by the Thomas procedure (33), are more truly "Pavlov pouches" than are those made by the classic Pavlov technique. Some investigators apply the term "Heidenhain pouch" to any type of fundic pouch deprived of its vagal nerve supply.

The completely innervated pouch has its widest use in studies on gastric secretory activities and is seldom employed in studies on gastric motor activities. Generally it is prepared to collect gastric juice and therefore includes the stomach areas which contain the most parietal and chief cells, i. e., corpus and fundus. Pouches have been used, however, for retaining certain substances or for bringing specific areas of gastric mucosa into contact with various chemical agents. An example is the pyloric antrum pouch used by numerous workers (see (3)) to demonstrate the gastrin mechanism. When a pouch of the fundus is made, it is advisable to establish a fistula of the main stomach also. This is extremely useful for introducing substances directly into the stomach, comparing the secretory activities of the pouch and main stomach, etc.

The area of the gastric mucosa incorporated in a Pavlov pouch should preferably not exceed $^1/_6$ of the total stomach area. This is approximately $^1/_5$ of the total acid-secreting capacity of the stomach. The larger the pouch the more gastric juice is lost and the greater the difficulties of maintaining the proper hydration and acid-base balance.

The fistula of the gastric pouch may be either cannulated or have the mouth brought out to the abdominal surface as a small mucosal roset. Cannulas, preferably of hard rubber, are useful in the Pavlov-type pouch, but for some unknown reason not in the Heidenhain-type pouch. For the Heidenhain pouch, a fistula with a narrow mouth gives the best results.

Several procedures have been described for making pouches with the express purpose of retaining the gastric juice for variable periods, among them the techniques of Goldberg and Mann (17) and Cope (11). But nearly all other types of pouches also may be made to function as retention pouches by simply making the pouch fistula narrow and bringing the fistula through the abdominal wall at an acute rather than a right angle. When the fistula is brought out at an acute angle and the animal is upright, the tension of the abdominal muscles results in a valvelike action which prevents leakage from the pouch. This is further aided by the constricting of the fistular orifice through formation of scar tissue. In some instances the latter process has been extensive enough to occlude the orifice and we have had to resort to surgery to reopen the pouch to relieve the distention caused by the accumulated secretions.

ESOPHAGEAL FISTULA

The operation for making an esophageal fistula in 1 stage is attended by a mortality of about 50%. Komarov (24) and Dragstedt and Mullenix (12) introduced a 2-stage technique which has proved successful. The following description is of the procedure as modified by us. It differs from those described by Komarov and Dragstedt in that the esophagus is covered by skin after the 1st stage of the operation and not pulled out over a bridge of skin and left exposed to the irritation of direct contact with bandages.

PROCEDURE

The esophagus is exposed by splitting the sternomastoid muscle somewhat to the left of the midline and, with great care to avoid injury to the blood supply, is freed from its bed for about 5 cm. The thin esophagus often is not readily identified: a helpful procedure at this stage is to pass a rubber tube into the esophagus through the mouth. The trachea is displaced slightly to the right and gentle traction exerted on the freed portion of the esophagus so that it is exteriorized as a loop. The split sternomastoid muscle is then reunited under the esophageal loop by several fine sutures to form a supporting floor for the esophagus. Both proximal and distal ends of the loop of exposed esophagus are sutured with fine 00 chromic catgut to the sternomastoid muscle, the suture being passed through only the muscle of the esophagus on the dorsal aspect (Fig. 1, *A*). The skin incision is then closed with the suture line running exactly over the esophageal loop. The esophagus should lie immediately below the skin; if necessary, excessive subcutaneous tissue should be trimmed away to achieve this (Fig. 1, *B*). One week later the original incision over the esophageal loop is reopened and the incision extended longitudinally through the anterior wall of the esophagus. The posterior esophageal wall is left intact. The skin margins and adhering esophagus may be cut away to leave an oval stoma about 3 cm long and less than 1 cm wide (Fig. 1, *C*). By omission of this last procedure and limitation of the fistula into the esophagus to a narrow slit, the continuity of the esophagus may be maintained for self-feeding purposes.

Feeding.—Animals with esophageal fistulas require special attention regarding food and water intake. The older procedure was to give food and water via the gastric fistula with which the animals are usually provided, but this is time-consuming and not entirely satisfactory. The procedure used by Komarov in Babkin's laboratory greatly simplifies the feeding problem. The stock diet consists of ground meat, porridge and milk. The animal is allowed to eat spontaneously; the material recovered from the esophageal fistula is refed several times so that sham-feeding persists about 3 min. The resulting excitation of the cephalic or initial reflex phase of digestive activity has been found to be of benefit in maintaining the animal's nutritional state.

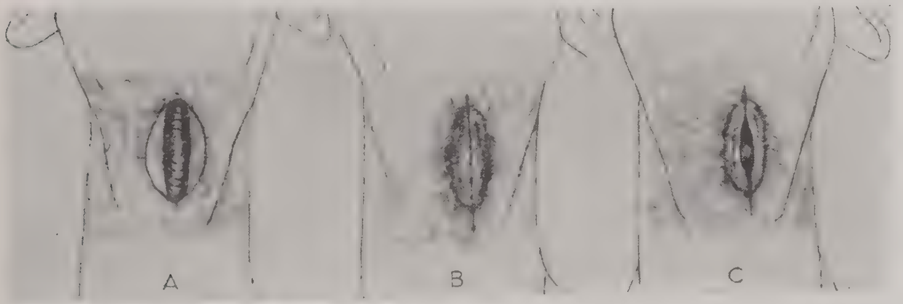

Fig. 1.—Stages in preparation of esophageal fistula. *A*, loop of esophagus exteriorized and supported by reuniting split sternomastoid muscle. *B*, esophageal loop covered by skin. *C*, fistula made by incision through skin and adhering anterior wall of esophagus below it. (Drawings, courtesy Hella Hochschild.)

The free end of 3 ft of rubber tubing to which is attached a large funnel is inserted in the stomach by way of the distal esophageal orifice. The tubing is slightly narrower than the diameter of the esophagus. When the tip of the tube reaches the cardiac sphincter it is carried into the stomach by gentle, slight pressure lasting about 1 min. The semiliquid food recovered from the proximal esophageal orifice during the sham-feeding period is placed in the funnel and "milked" into the stomach by squeezing the contents along the rubber tube. The stomach is filled in about 5 min and the tube then withdrawn. When necessary, water is similarly administered by this route.

In animals with the esophageal fistula limited to a narrow slit, feeding may sometimes be simplified by approximating the lateral walls of the severed esophagus and skin by several clips. Serrafines or so-called bull-dog artery clips are satisfactory. Usually the animal can then ingest and pass down the esophagus most of a solid meal, but liquid foods usually leak through the fistula and may necessitate administration as already described.

Complications.—The major difficulties usually encountered are mediastinitis and esophageal obstruction and rupture. By restricting the dissections to the immediate operating field and avoiding the splitting of muscle sheaths as much as possible one can greatly reduce the incidence of mediastinitis. Obstruction is usually due to placing the esophagus under too great tension and having the sternomastoid muscle fit too tightly. The poor blood supply to the esophagus makes it particularly vulnerable to devascularization and hence susceptible to perforation.

GASTRIC POUCH FOR ACUTE EXPERIMENTS

A gastric pouch suitable for acute experiments may be made by simply isolating the stomach between ligatures placed at the cardia and the pylorus and establishing a gastrostomy of the corpus. If it is desired to study the effect of vagus stimulation, the ligature at the cardia is either omitted or placed around the cervical esophagus. The stomach also may be subdivided into 2 pouches, for example, 1 of the antrum and the other of the corpus, by means of a rubber stopper secured between the areas.

PROCEDURE

Through a small incision in the anterior wall at the angularis the mucosa is separated from the muscularis by a ligature-carrying probe. The probe is passed between the coats until the separation circumscribes the stomach and the ligature can move freely in the tunnel thus made. The rubber stopper, with a groove cut around the edge, is then slipped into the stomach and, after it becomes properly situated, is tied in place by tightening the ligature around the stopper groove. Since the ligature passes only around the mucosa, the innervation of both pouches remains intact. The same procedure may, of course, be used to divide any other part of the stomach or intestine with minimal interruption of the nerve supply.

Usually the gastrostomy for recovering the juice is a cannulated fistula of the anterior wall. However, if it is desirable not to section the anterior gastric wall, the pouch may be drained through a hard rubber tube inserted through the pylorus.

This type of whole stomach pouch has proved useful in studies on humoral secretagogues and in certain histophysiologic studies of the gastric glands. It is not suitable for studies involving reflex phases of gastric secretion, particularly when barbiturates are used.

INNERVATED GASTRIC POUCH

Chronic gastric pouches may be divided broadly according to the gastric region or type of mucosa incorporated and the integrity of the extrinsic autonomic nerve supply. The choice of pouch is determined mainly by the nature of the experiment. In limited space we can devote attention to only a few which we may consider representative of the several types. Since the nerve supply to the pouch is a major factor, it is desirable first to describe briefly the innervation of the stomach in the dog.

Innervation of stomach.—Immediately below the level of the tracheal bifurcation, the right and left vagus nerves lying on the lateral walls of the esophagus and invested in the mediastinal pleura divide into 2 branches, ventral and dorsal. The anterior branch of the left vagus is usually larger than the posterior branch and the posterior branch of the right vagus is usually larger than the anterior branch. The posterior branches of both nerves unite dorsal to the esophagus just above the diaphragm to form a large dorsal trunk, and the anterior branches similarly join to form a ventral trunk. Each trunk is thus a mixture of fibers derived from both vagus nerves (26, 32). The 2 trunks pass through the hiatus of the diaphragm on the ventral and dorsal sides of the esophagus and maintain this relation to the stomach as they continue parallel to the lesser curvature. Branches from the ventral* trunk supply the entire anterior part of the stomach, pyloric sphincter, duodenum and liver. The dorsal trunk supplies the entire corresponding posterior side of the stomach and also sends a branch to the celiac plexus.

The vagus trunks which run along the lesser curvature send their fibers over the anterior and posterior walls of the stomach essentially at right angles to the lesser curvature. The fibers at first lie superficial to the mucosa and only near their terminations turn in toward the mucosa. Since an isolated pouch made from the fundus high in the greater curvature and near the cardia behaves essentially like a fully innervated pouch, this region of the stomach probably also receives vagus fibers by way of the blood vessels and the greater omentum. This point was utilized by Brestkin and Savitch (9) in making a Pavlov pouch by the Heidenhain procedure and indicates that not all Heidenhain pouches are truly vagally denervated.

---

* The designations "ventral" and "dorsal" are preferred to "left" and "right" as used by anatomists because the inference is inherent in the latter terms that the "right" and "left" nerves in the abdomen represent the corresponding nerves in the cervical region. Bradley *et al.* (J.A.M.A. 133: 459, Feb. 15, 1947) proposed the terms "left gastric nerve" and "right gastric nerve" for the vagus nerve trunks which have passed through the hiatus of the diaphragm to the stomach.

The sympathetic nerve fibers supplying the stomach originate in the celiac ganglion and reach the stomach mainly with the gastric arteries. Most fibers accompany the left gastric artery, but those to the cardia proceed mainly with the inferior phrenic arteries. The pylorus is also supplied by fibers which descend from the hepatic artery. Some sympathetic fibers also pass to the stomach with the pyloric artery and right gastroepiploic artery (27). Some sympathetic fibers also may be distributed to the stomach with the vagal fibers.

TOTAL STOMACH POUCH

In general, pouches of the total stomach may be the vagally denervated type prepared by the techniques of Frémont or Ivy or the vagally innervated type prepared by the technique of Dragstedt and co-workers. The latter has the advantage that denervation may be carried out subsequently.

In the Dragstedt (13) operation the stomach is exposed through a long midline incision and the suspensory ligament of the pylorus divided. The spleen and omentum are delivered from the peritoneal cavity, the upper part of the stomach being retracted down and the liver to the right. Through an incision high in the omentum at the greater curvature of the stomach the index finger is passed and forced around the esophagus. The blood vessels are similarly underpassed. Nerves and blood vessels are further displaced by gentle traction so that a wide vessel- and nerve-free ring is established at the esophagogastric junction. Two rubber-shod clamps having right-angle jaws are placed on the esophagus and stomach, respectively, and while gentle traction is exerted on these clamps the esophagus between them is transected. Care is observed to leave the vagus nerves essentially undamaged. The stomach is then divided between clamps at the pylorus. The lower end of the esophagus is united with the open end of the duodenum by an end-to-end anastomosis. If the duodenal orifice is narrower than the esophageal, the end of the esophagus may be united to the side of the upper duodenum and the end of the duodenum is closed.

The cardiac and pyloric ends of the stomach are inverted and closed by 2 rows of sutures. A gastric cannula of suitable material (Lucite, Nylon, silver, stainless steel, gold-plated brass) is inserted in the body of the stomach at the anterior surface near the greater curvature and brought out through the abdominal wall. The suture lines in the stomach are reinforced by wrapping some omentum across them and around the stoma through which the cannula passes (Fig. 2).

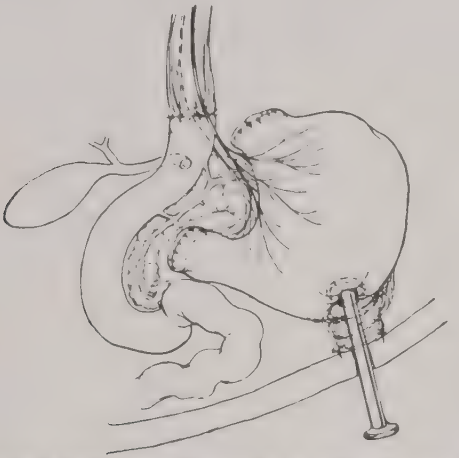

FIG. 2.—Dragstedt total stomach pouch with preservation of blood and vagus nerve supply. (From Dragstedt *et al.:* Arch. Surg. 60: 1, 1950.)

PAVLOV FUNDUS POUCH

The anatomic basis for the technique used by Pavlov (30) is now known to be erroneous, but there is evidence that both Pavlov and his pupils became aware of this long before Jemerin and Hollander (21) called attention to this point (2). Although when the classic Pavlov pouch is made, many of the vagus fibers are interrupted, sufficient vagal innervation usually remains to permit significant studies on the nervous control of gastric secretion.† In time, however, many of the nerve fibers to the pouch apparently degenerate since in some animals the pouch then becomes indistinguishable from a Heidenhain or vagally denervated pouch.

A classic Pavlov pouch is prepared by opening the abdomen by a midline incision and delivering the stomach onto saline-moistened towels which border the wound. The gastroepiploic artery on the greater curvature at a point approximately $^2/_3$ distal from the cardia is divided between ligatures. The omentum is cut and small blood vessels are ligated so that a devascularized area is made 1 cm wide on the greater curvature. Through this space 2 gastric clamps are introduced around the stomach and passed approximately parallel to the greater curvature. The clamps are extended cephalad about $^2/_3$ of the way across the stomach (Fig. 3, *A*), so that when fixed they separate the stomach along the line *ab* but not along *bc*. The clamps are kept about 1 cm apart and all coats of both walls of the stomach are cut between the clamps. The incision must not extend beyond point *b*. Hemostatic clamps are introduced at *b* and the gastric clamp on the pouch is removed. Hemostasis is obtained by small clamps applied to necessary points on the edge of the severed wall. The gastric clamp on the stomach is moved back from the edge toward the lesser curvature to act as a hemostatic clamp.

---

† Pavlov and his associates considered a pouch to be vagally innervated or denervated on the basis of functional criteria rather than anatomy. The pouch was considered to be supplied with vagus fibers only if it reproduced exactly, but in miniature, the secretory picture of the main stomach. For this reason all animals with gastric pouches were also equipped with cannulated fistulas of the stomach.

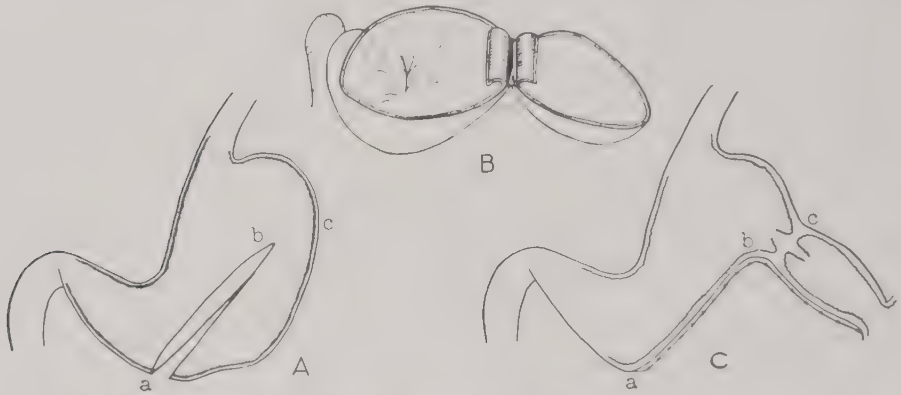

Fig. 3.—Preparation of vagally innervated gastric pouch by Pavlov technique. *A*, all layers of anterior and posterior wall sectioned along line *ab*. *B*, as viewed from greater curvature: only mucosa along line *bc* is cut through to form a double mucosal septum. *C*, completed pouch, showing double wall of mucosa separating pouch from main stomach. (Fig. 3, *B*, adapted from LePlay *Technique operatoire physiologique* [Paris: 1912].)

The isolated part of the stomach is now pulled back to form an everted flap hinged along *bc*. With a sharp scalpel an incision is made through the mucosa of the anterior and posterior walls at *bc*. The pouch thus remains connected to the main stomach by the seromuscular layers only and the pouch mucosa is entirely separated from the main stomach mucosa (Fig. 3, *B*).

The flap is now rolled into a tube. Before this, the mucosa at the base is inverted into the tube and kept in position by through-

and-through sutures to form an impervious septum of mucosa across the base of the tube. The edges of the tube are now approximated and, with the edges inverted, sewn together by a continuous suture which begins at the base and extends to but does not include the tip of the tube. Several reinforcing sutures may be taken also. The main stomach is now completely closed. It is important that the cut mucosa on the main stomach side of the "hinge" be brought together to form a 2d mucosal septum across the base of the pouch (Fig. 3, *C*). The open tip of the tube which now constitutes the pouch is brought out on the skin surface through a small stab wound and fixed in place by suturing the tip of the pouch to the peritoneum, muscle and fascia.

HOLLANDER FUNDUS POUCH

Dissatisfaction with the classic Pavlov pouch led Hollander and Jemerin (19) to modify the original procedure so as to conserve the vagal innervation to a greater degree. In their technique, the stomach is delivered through an upper abdominal incision to the left of the midline. A single gastric clamp is applied parallel to its long axis and across both fundus and pyloric antrum close to the lesser curvature. The initial incision in the stomach starts on the greater curvature, about 1 in. proximal to the incisura angularis (Fig. 4). From this point it extends about halfway across both the anterior and the posterior stomach wall in a direction transverse to the long axis of the stomach. The incision is made through the entire thickness of both gastric walls. The gastroepiploic vessels can usually be separated bluntly from the greater curvature far enough to permit the incision. A point on the greater curvature of the fundus about equidistant between the cardiac and the most

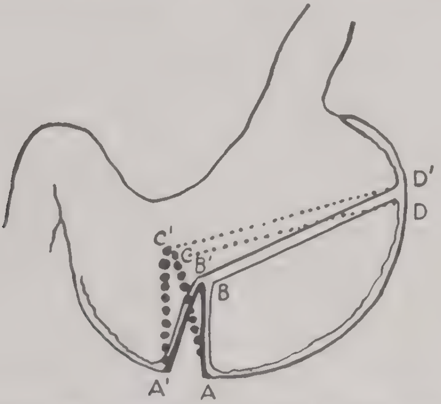

Fig. 4.—Hollander-Jemerin modification of Pavlov technique. (From Hollander and Jemerin (19).)

dependent portion of the stomach is selected and a long threaded suture is passed from without inward. Then by traction on the suture the attached fundus may be everted through the incision. The assistant's finger is placed under the serosal aspect of the line *B–D*, exposing the mucosal side; the mucosal corrugations are eliminated by properly applied tension. With a very sharp scalpel the mucosa is incised along *B–D* and, without releasing the position or tension, flaps of mucosa about $^3/_8$ in. wide are dissected back on either side of the incision by sharply splitting the submucosa. Care must be taken not to traumatize or shred the mucosa or to dissect deeper than the submucosa. The same process is repeated along *C–D*, thus completing the mucosal incision from *B* to *D* to *C* without interrupting seromuscular continuity, so that most of the branches of the vagus nerve supplying the pouch continue to pass uninjured from the main stomach onto the pouch. The mucosal flap of the anterior wall of the pouch is sewn to the mucosal flap of the posterior wall of the pouch, begun at the greater curvature with a running inverting suture of fine silk or linen. Similarly the anterior and posterior flaps of mucosa of the walls of the main stomach are sutured together. This results in separation of the stomach from the pouch by a double-layered septum of mucous membrane. Sutures should be small and closely spaced.

Closure of the stomach is now completed by suturing *B′–A′* to *C′–A′*; *B–A* is sewed to *C–A*, leaving, however, an opening at *A* for the pouch mouth. We prefer to have the fistula no larger than sufficient to admit a no. 16 F catheter.

A small counterincision is made in the abdominal wall, to the left of the main wound, at the site where the pouch can be brought up with minimal tension. Skin and fascia are incised and muscle is separated bluntly. After the peritoneum is incised the pouch mouth is brought up through the counterincision and sutured to the peritoneum, fascia and skin. The small size of the pouch stoma, added to the natural tonus of the bluntly separated musculature through which it passes, usually results in sufficient sphincter action to prevent escape of gastric juice from the pouch. The main wound is closed in layers.

NECHELES FUNDUS POUCH

Several investigators found the Hollander pouch difficult to make and maintain without rupture of the mucosal bridge, and Neuwelt, Olson and Necheles (29) were led to modify the older Boldyreff (8) technique. It is less difficult than the Hollander-Jemerin procedure, but the section through the length of the anterior wall of the stomach leaves intact only about 50% of the vagal supply to the pouch.

The stomach is exposed and the blood vessels at the greater curvature below the angularis are ligated and cut. Rubber-covered elastic clamps are put across the lower third of the fundus. A straight incision is made through the entire gastric wall (Fig. 5). The exposed mucosa of the posterior wall is cut and about 1 cm is dissected away from the muscularis on each side of the incision. The main stomach is closed by an inverting running suture of the mucosa. The mucosa of the pouch is similarly closed, beginning at the upper end of the incision. The elastic clamp is removed when about half the pouch is closed. At the lower pole of the pouch a cannula is inserted and the submucosal suture carried in such a way as to keep the base plate of the cannula away from the mucosal septum and so avoid perforation between pouch and main stomach. The muscularis of the pouch and of the main stomach is united by Lembert sutures. Omentum is wrapped around the cannula base. A small skin incision is made to the right of the midline at the height of the greater gastric curvature, a sharply pointed trocar with sleeve is pushed through it, the trocar withdrawn, the cannula pushed into the sleeve and the sleeve withdrawn. The abdomen is closed in the usual way.

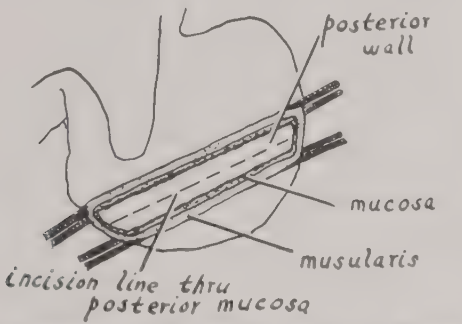

Fig. 5.—Stage in preparation of gastric pouch by Neuwelt-Olson-Necheles modification of Pavlov technique. The posterior wall mucosa is exposed by incision carried through all layers of entire anterior gastric wall; posterior wall mucosa only is then incised along line shown. (Modified from Neuwelt *et al.* (29).)

COPE FUNDUS POUCH

The 2-stage operation for formation of a pouch described by Cope *et al.* (11) conserves much of the vagus innervation.

First stage.—The stomach is withdrawn through a midline incision and fixed by stay sutures at points *a*, *b*, *c* and *d* (Fig. 6, *A*).

With thermoelectric cautery an incision through the posterior wall of the stomach is made close to and parallel to the greater curvature along *cd*. It is extended across the anterior wall of the antrum along *dr* and through the anterior wall along *rs* which runs parallel to, but some distance from, the lesser curvature. This results in a flap of anterior wall which is retracted cephalad to expose the gastric mucosa. The anterior wall along *sc* forms the hinge for the retracted flap. By electric cautery an incision is made through only the mucosa of the anterior wall connecting points *s* and *c*. By continuous inverting Connell sutures with 00 chromic catgut, the greater curvature of the anterior wall is approximated to the lesser curvature. The pyloric end, however, is left open to form a rolled-up tube of the anterior gastric wall (Fig. 6, *B*). The mucosa which was sectioned along the hinge line *sc* is converted into 2 septums by suturing the mucosal edges after they have been inverted into the lumens of the main stomach and the pouch, respectively. The closing sutures are made from the top down, with

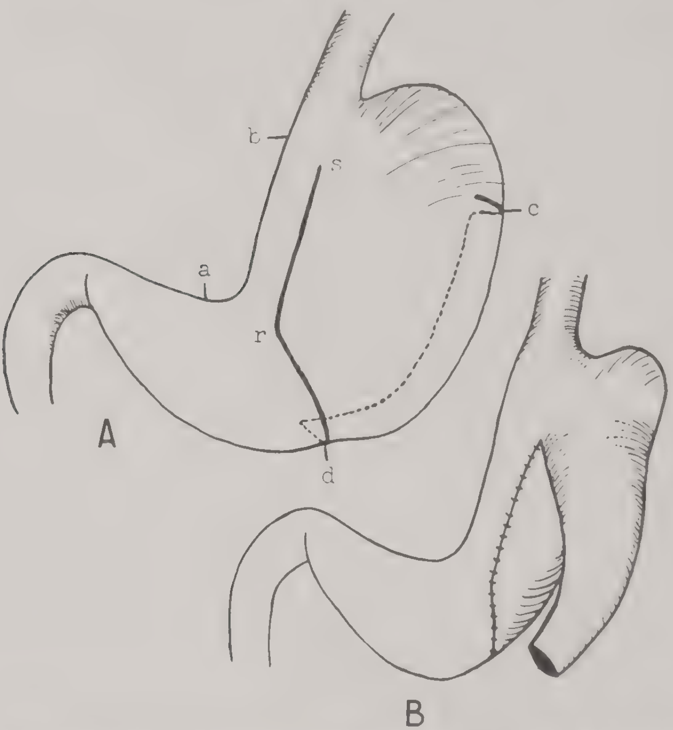

Fig. 6.—Gastric pouch by technique of Cope *et al.* *A*, lines of incision through posterior wall (broken line) and anterior wall (solid line). *B*, completed pouch before stoma is brought into antrum (see text). Mucosal septum at base of pouch not shown. (Drawings courtesy Hella Hochschild.)

the open end as far from the esophagus as possible. Similarly the greater curvature and the lesser curvature of the posterior gastric wall are approximated to form the main stomach. Care is taken to avoid stricture formation in the main stomach especially at the cardiac and pyloric ends. The rim of the open end of the pouch, which consists of muscle from the anterior gastric wall, rather than the edge of the greater curvature, is then sutured into an opening 2.5 cm in diameter in the anterior wall of the pyloric antrum. Thus a pouch of the anterior gastric wall results which drains into the main stomach at the antrum. No difficulties appear to be encountered by retrograde flow of food into the blind pouch.

Second stage.—About 3 weeks later, through a lateral left rectus incision, the antral end of the pouch and a 1 cm wide cuff of antral wall (serosa and muscularis only) is excised and transferred from the antrum to the left flank. The spleen swings with the pouch. The antrum incision is closed by sutures and covered with omentum. The cuff of antral wall is brought through a stab wound in the left flank and sutured loosely over the skin. The pouch wall is sutured to subcutaneous fascia with the fine chromic catgut sutures. It is important that the pouch be brought out posteriorly on the flank in a position which causes no tension.

The properly made pouch has a sphincteric action due to the antral cuff and the abdominal wall. Sphincteric action is maintained only with the animal in the upright position or lying on the right side. The gastric contents will not be retained when the animal lies on the left side. Therefore, for experiments requiring retention of the secretion over a period of time, the animal must be trained not to lie on its left side.

BRUNSCHWIG SUBTOTAL POUCH

Using a technique essentially similar to that of Dragstedt, Brunschwig and co-workers (10) prepared a subtotal gastric pouch which includes the fundus and corpus but not the antrum. The stomach is transected at the cardiac end, with care not to sever the vagal fibers. The corpus is separated from the antrum and continuity of the gastrointestinal tract is re-established by end-to-end anastomosis between esophagus and antrum. The discrepancy between the large antral orifice and the small esophageal orifice is corrected by partial closure of the antrum with sutures. The cardiac and antral ends of the pouch are closed by inversion and if possible sealed with omentum. A gastric cannula is inserted in the pouch in the usual manner.

The vagal fibers supplying this pouch remain intact, but those coursing to the antrum via the gastric walls are of course severed.

Establishment of a cannulated fistula into the antrum to permit direct introduction of substances makes the preparation suitable for study of both nervous (initial reflex) and humoral (pyloric and intestinal) components of the gastric secretory mechanism.

THOMAS FUNDUS POUCH

The method developed by Thomas (33) retains maximal vagal innervation. The pouch has had thorough trials in our laboratory over several years and is recommended as more suitable and reliable than those hitherto described, particularly for investigation of problems of reflex gastric secretion.

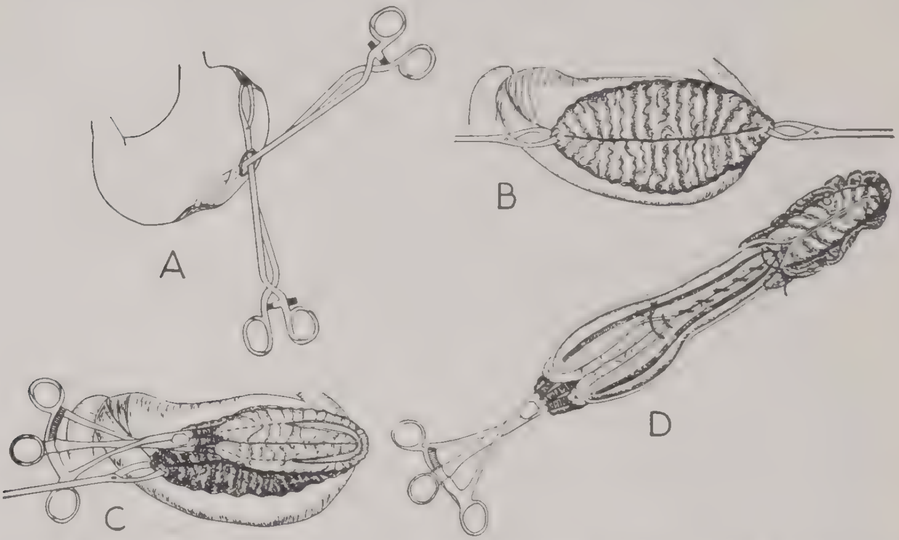

Fig. 7.—Stages in preparation of fully innervated gastric pouch by Thomas technique. *A*, mucosa grasped at 2 points by vulsellum forceps inserted through small stomach wall incision near greater curvature. *B*, portion of stomach destined to become pouch turned inside out. *C*, 1st position of 3-bladed gastroenterostomy forceps; mucosal incision made at upper end and to be extended along broken line. *D*, several steps in suturing: *lower left*, flaps prepared for suturing; *center*, suturing of inner (main stomach) mucosal flaps to form 1 septum; *upper right*, suturing of outer (or pouch) mucosal flaps to form other septum. (Modified from Thomas (33).)

The fundic portion of the stomach is delivered through an abdominal incision in the usual manner and the site of the pouch selected. A 2 cm incision is made in the center of the pouch area, crossing the greater curvature at a right angle and through the entire gastric wall including the mucosa. Two pairs of vulsellum forceps are inserted through the incision and used to grasp the mucosa at the 2 points destined to be the extremities of the pouch (Figs. 7, *A*, and 8, *A*). The entire pouch area is everted through the incision by gentle traction on the vulsellum forceps and hand manipulation of the stomach wall. Further traction on the forceps, in opposite directions, pulls the circular rosette of everted mucosa into an oblong shape so that 2 roughly parallel ridges of mucosa appear stretched between the forceps (Fig. 7, *B*).

A 3-jawed gastroenterostomy forceps is applied so that the center jaw lies between the ridges of mucosa with its tip near the attachment of 1 pair of vulsellum forceps and as great a length as possible of each mucosal ridge is grasped between the center jaw and the corresponding outer jaw (Fig. 7, *C*). Even a large pair of forceps will be too small to encompass the whole distance between the pairs of vulsellum forceps. For this reason, when the procedures described below are completed in 1 area the gastroenterostomy forceps is shifted to the next adjacent area and so on until the entire distance has been covered. With the gastroenterostomy forceps in position, a hairpin-shaped incision is made through the mucosa only, extending along the crest of each ridge of mucosa and around the end where the vulsellum forceps, which are now removed, were attached (Fig. 7, *C*). This incision is eventually to be extended to encircle the pouch area, but·is temporarily continued only to where the mucosal ridges disappear between the blades of the gastroenterostomy forceps.

The mucosa along each side of the incision is undercut with iris scissors to form mucosal flaps about 1 cm wide (Fig. 8, *B*). The 2 inner flaps of mucosa are sutured together so as to approximate the submucosal surfaces and turn the edges in toward the main stomach (Figs. 7, *D*, midportion, and 8, *C*). The outer flaps are sutured in like manner but with the edges turned out (Figs. 7, *D*, upper right end, and 8, *C*). The suture lines are continued as far as possible, the inner a little farther than the outer, and the forceps then shifted toward the other end of the pouch. Incisions and suture lines are continued as before, shifting the forceps whenever necessary until the suture lines are within a few centimeters of the second pair of vulsellum forceps, which is still attached to the mucosa. All forceps are now removed and the incisions and suture lines continued until they meet where the ridges of mucosa join at the end of the pouch. Thus a double mucosal wall has been constructed between pouch and main stomach. The pouch is now turned right side out and the original small gastric incision closed with a double row of sutures. This provides a pouch fully innervated by the vagal fibers passing to it over the muscularis and yet separated from the main stomach by a double layer of mucosa (Fig. 8, *D*).

A hard rubber (vulcanite) cannula is inserted at 1 end of the pouch at the point judged to be the most dependent when the animal is upright. Insertion of the cannula through the original gastric incision may be preferable, in which case the incision should be made near 1 end rather than in the center of the pouch area. After the stomach has been returned to its natural position in the abdominal cavity the outer end of the cannula is brought out through a stab wound in the abdominal wall, so located as to cause minimal displacement of the stomach. A soft rubber drain tube is inserted in the pouch via the cannula and retained by a thread passed through small holes drilled diametrically opposite each other through the cannula near its outer end.

Reinforcing sutures placed at intervals through both gastric walls in the area between the mucosal bridges (Fig. 8, *C*) have been

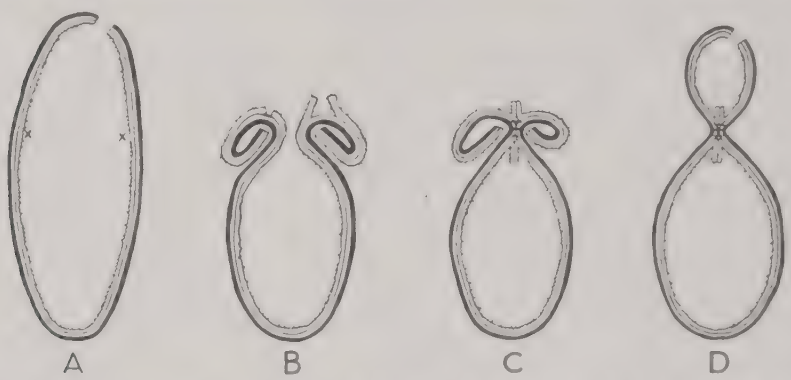

Fig. 8.—Diagrammatic scheme of stages in preparation of Thomas pouch: greater curvature at top; anterior wall at right. *A*, small incision through stomach wall; mucosa to be grasped by vulsellum forceps at *X*. *B*, part of stomach everted through incision with crest of mucosa only cut (left); this is then undercut (right) to make 1 cm wide mucosal flaps. *C*, sutures through submucosa at base of mucosal flaps and reinforcing submucosal suture. *D*, completed pouch after turning right side out.

found to reduce incidence of septum perforation. Some difficulty is sometimes experienced, however, in inverting the mucosal edges properly. The technique described by Watman and Nasset (34) overcomes this difficulty and probably also provides greater security in this area. A ribbon of mucosa about 0.5 cm wide is excised from the crest of the rosette with curved scissors. This obviates the undercutting of the mucosa described originally by Thomas (shown in Fig. 8, *B*, right side). The innermost mucosal layer (of the gastric lumen proper) is brought together with a Connell suture so that the raw surfaces are apposed, and the submucosa is brought together with a running mattress suture and a few reinforcing interrupted mattress stitches. Finally, the outer mucosal layer (of the pouch) is closed with a running mattress suture which apposes the raw surfaces.

VAGALLY DENERVATED GASTRIC POUCH

TOTAL STOMACH POUCH

Frémont and Ivy pouches are prepared essentially as the Dragstedt pouch, but the vagus nerves to the stomach are completely severed when the esophagus is separated from the stomach. In the Frémont pouch the gastric cannula is inserted near the greater curvature after the cardiac and pyloric ends of the stomach are closed. In the Ivy pouch only the cardiac end is closed and the pyloric end of the stomach is drawn through a stab wound in the abdominal muscles and skin. This permits the gastric juice to drain through the pyloric stoma.

In dogs with a sufficiently long subdiaphragmatic esophagus the duodenum can be brought up to the esophagus and an esophagoduodenal side-to-side anastomosis established before the esophagus is divided from the stomach. This is advantageous since the stomach may be used for exerting traction. However, the stump of esophagus below the esophago-duodenal stoma may form a blind pouch which ruptures readily. This type of preparation at best may be regarded as a semichronic preparation and therefore an end-to-end esophagoduodenal anastomosis should be made whenever possible.

When it is impossible or undesirable to produce an esophagoduodenal anastomosis the stomach may be completely isolated from the esophagus and duodenum and equipped with a gastric cannula. An esophageal fistula is made and the distal end of the esophagus closed. A cannulated fistula is also made in the jejunum to permit feeding. Animals may be kept in good condition for several months, but require close attention (35).

HEIDENHAIN POUCH

Probably the simplest and most widely used gastric pouch is that introduced by Heidenhain (18). It was the first isolated gastric pouch, and others made since are modifications of it. The operation consists of excision from the stomach body of a segment usually including the greater curvature and retaining the blood supply intact. This segment is converted into a miniature stomach which drains to the outside.

The right gastroepiploic artery is tied off on the greater curvature several centimeters proximal to the antrum. Small vessels are divided between ligatures for a distance of about 1 cm. Two rubber-shod clamps are introduced through this devascularized area and directed cephalid to divide the stomach lengthwise. This results in a gastric segment composed of the greater curvature and anterior and posterior walls of part of the corpus and fundus. All layers of the anterior and posterior walls are sectioned by scissors passed between the clamps.

The clamp on the main stomach is moved back from the edge and the stomach incision closed: a single continuous suture inverting through all coats is sufficient. The clamp is now removed and the incision line covered with omentum. The clamp on the segment is moved back from the edge and the segment formed into a pouch by inverting sutures which commence at the cardiac end and pass through all layers. The distal or pyloric end of the pouch, however, is left open as a narrow mouth of the pouch. A stab wound is made on the left side of the abdominal wall near the midline and the mouth of the pouch is drawn through it. The pouch neck is fixed to the peritoneum and muscle to leave only a small segment of mucosa extruding as a rosette on the abdominal surface (Fig. 9). The abdomen is closed in layers. Accumulation of blood and secretions in the pouch is prevented by a soft rubber catheter left for 24 hr.

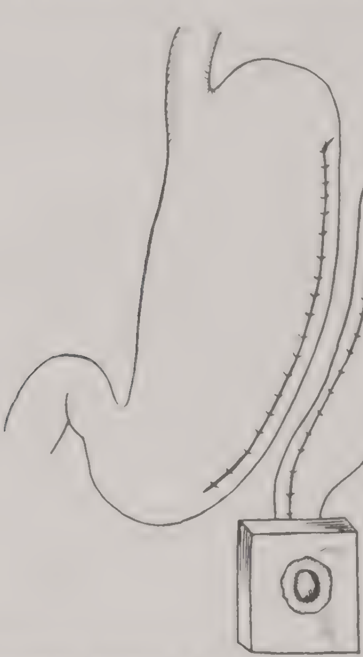

FIG. 9.—Heidenhain pouch of body without vagal innervation.

A pouch containing the fundus mucosa adjacent to the cardia behaves like a vagally innervated Pavlov pouch (9). Apparently this is due to vagal fibers contained in the greater omentum. Probably the degree of vagal innervation of the Heidenhain pouch varies with the amount of proximal fundus tissue incorporated, but even when the pouch is made with an attempt to exclude this critical area it is not uncommon to find persistence of vagal innervation (25). It is therefore advisable that a test (e.g., with insulin) be performed in each instance to determine the presence or absence of functional vagus fibers.

Comment by J. P. Quigley

In studies which demonstrated that insulin produces motor inhibition of the vagotomized stomach and augmentation of the normal stomach we (Am. J. Physiol. 91: 467 and 482, 1930) observed, as have most investigators, that the usual Heidenhain pouch made from the gastric corpus is invariably vagally denervated.

GASTRIC POUCH OF GOLDBERG AND MANN

Goldberg and Mann (17) devised a 2-stage procedure for making the pouch leakproof. In stage 1 a Mann-Bollman (28) fistula of the fundus is made. A loop of terminal ileum 10–15 cm long is resected with blood supply intact and intestinal continuity reestablished by end-to-end suture. The distal end of the resected loop is sutured to an opening made in the anterior wall of the fundus near the greater curvature and the proximal end is brought through a stab wound to the skin surface, where it is fixed. The gastrostomy opening should be a little narrower than the diameter of the intestine sutured to it. The abdomen is closed in layers. In 3–4 weeks the abdomen is reopened through the same incision and the gastric fundus with its attached intestinal segment exposed. This portion of the stomach is now made into a pouch in the usual manner. The entire pouch is closed; the intestinal loop drains the pouch contents to the exterior.

TRANSPLANTED POUCH OF IVY AND FARRELL

For studies on the humoral mechanism of gastric secretion Ivy and Farrell (20) transplanted a gastric fundic pouch in a 2-stage operation. The transplantation preferably is made in female dogs that have recently whelped so that the mammary gland beneath which the pouch is to be planted is assured a good blood supply. In stage 1 a Heidenhain type pouch about 8 cm long and 5 cm in diameter is made as usual. An incision is made through the skin of the mammary gland closest to the pouch area. By blunt dissection a cavity is made beneath the mammary tissue. It is made to communicate with the abdominal cavity by a stab wound through the remaining abdominal wall. The pouch is drawn through the stab wound and brought to lie beneath the cavity of mammary tissue. The pouch mouth is brought to the skin surface. Traction on the pouch must be avoided since it is essential that the mesenteric vessels supplying it be left intact. The stab wound is loosely closed around the pouch pedicle and the skin incision loosely closed around the pouch mouth.

Two to 4 weeks later, preferably when the dog is in heat, the abdomen is opened by an incision lateral to the pouch orifice and the pedicle of the pouch is located. This is cut between ligatures and peritonealized and the abdominal incision closed in the usual manner. The pouch is now entirely freed from its original vascular and nervous connections and is maintained by blood vessels (but not nerves) that have grown into it from the region into which it was transplanted.

KLEIN POUCH WITHOUT AUERBACH'S PLEXUS

When it is necessary to consider the role of Auerbach's plexus as well as extrinsic innervation, a secreting pouch made by Klein (22) offers some advantages. A small Heidenhain type pouch of the fundus is made as usual. The blood vessels to the pouch enter along the greater curvature and pass almost directly to the submucosa. With iris scissors an incision is circumscribed through the muscularis only, with care not to cut through the submucosa or puncture the mucosa. The edge of the cut muscularis is lifted with forceps and undercut with scissors. This is continued until the whole muscularis has been peeled off, leaving only a pouch of mucosa and submucosa. Occasionally the muscularis may be peeled off by gentle traction with the fingers, if the cut edges of muscle are held with saline-moistened gauze to increase the traction grip. After the muscularis has been removed, the submucosa is scraped with a scalpel to insure against chance adherence of muscle fibers.

The pouch is brought through a stab wound in the abdominal wall into a sac prepared for it in the subcutaneous tissue. Traction on the pedicle is avoided. Several retention sutures are carefully placed in the pouch and the skin around the mouth of the sac is closed loosely. In 4–6 weeks the pouch has established new vascular connections and the pedicle of blood vessels and nerves to the pouch may be severed through an abdominal incision.

This pouch is completely without vagus and sympathetic innervation and without Auerbach's nerve plexus. If required, much of the submucosal plexus may be destroyed by painting the walls with silver nitrate solution. Because the pouch has no muscular coat it does not empty by itself; secretion is removed entirely by gravity drainage or suction through a catheter.

SPECIAL POUCHES

Various modifications of the more important pouches have been developed to meet special requirements.

Completely denervated pouch.—In an attempt to produce a pouch deprived of both sympathetic and parasympathetic innervation, Bickel and Katsch (6) stripped the walls of the arteries supplying the pouch. After a Heidenhain pouch was made and all or most of the vagal innervation was destroyed, the entire omentum was severed and periarterial sympathectomy performed by stripping the blood vessels supplying the pouch. Only about 10% of animals are said to survive such drastic procedure, and the resulting pouch is apparently in no way superior to the transplanted pouch made by the Ivy-Farrell technique.

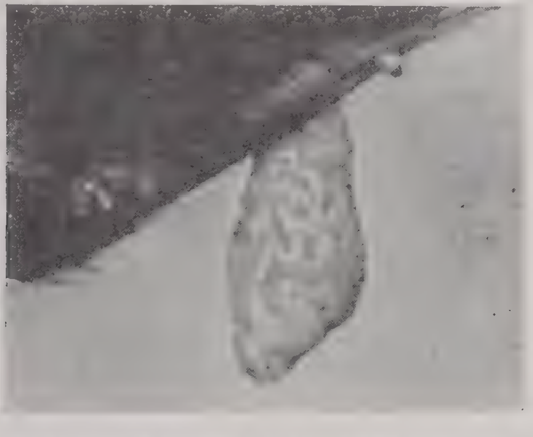

FIG. 10.—Everted fundus pouch by Friedman technique. Picture (from 16 mm color film) taken 6 weeks after operation shows small area of edema near tip of pouch.

Everted fundus pouch.—A pouch devised to demonstrate the effect of direct application of various agents to the mucosal surface (15) is equally satisfactory for study of the processes of gastric ulceration and desquamation of gastric epithelium. It is essentially a short Heidenhain pouch prepared with a wide mouth. The day after operation the base of the pouch is grasped by vulsullum forceps inserted through the pouch mouth and retracted gently so that the pouch becomes wholly everted. Care is observed not to rotate the pouch around its long axis since this results in twisting of its pedicle and blood supply. The everted pouch is kept covered with petroleum jelly and a bandage for about a week. Such an appendage of everted gastric mucosa with intact blood supply is capable of secreting gastric juice in response to appropriate humoral stimuli (Fig. 10). When not in use the pouch may be gently pressed back into the abdominal cavity and the abdominal bandage restored. However, a small pouch may be left everted indefinitely if the animal is permitted to lick the appendage at will.

Lesser curvature pouch.—To study specifically the secretion from the lesser curvature of the stomach Armour (1) made a pouch from this area which retains its vagal innervation. This region is a common site of gastric lesions, and Armour felt that information about its secretory activities would be of special interest.

A horseshoe-shaped incision is made through the anterior wall of the stomach in such a way that the prongs begin about 2 cm from the lesser curvature and encompass about a third of the fundus. The flap thus formed is lifted and a similar horseshoe-shaped incision is made through only the mucosa of the exposed posterior wall, beginning at the points where the incision through the anterior wall terminate and of identical contour. Throughout the length of the 2 horseshoe incisions the mucosa is freed from the seromuscular wall about 1 cm from the margin.

The flap is rolled to form cone-shaped pouch and the mucosa at the base of the cone is formed into a septum as in the usual Pavlov pouch. The mucosa of the approximated edges is sewn together and also the edges of the seromuscular layers. At the base of the pouch on the side of the main stomach the mucosa is united as a wall and the rest of the stomach closed in the usual manner. The stomach becomes slightly distorted when the defect in the lesser curvature resulting from exclusion of the pouch area is repaired. The mouth of the pouch is brought out through a stab wound in the abdominal wall.

Comment by J. P. Quigley

Unusual gastric pouches or other stomach preparations have been devised for special purposes by many investigators; e.g., Grossman, M. I., *et al.:* Am. J. Physiol. 153: 1, 1948; Woodward, E. R., *et al.:* ibid. 162: 99, 1950; Lim, R. K. S., and Mozer, P.: Report at the meeting of the American Physiological Society, Columbus, O., September, 1950; Quigley, J. P., and Templeton, R. D.: Am. J. Physiol. 91: 475, 1930; Quigley, J. P., *et al.:* ibid. 108: 643, 1934.

POSTOPERATIVE CARE

The major causes of early postoperative death following gastrointestinal surgery are: (1) inadequate blood supply to a viscus, leading to gangrene; (2) improper approximation of anastomoses, leading to perforations; (3) local tissue strangulation by sutures tied too tightly; (4) shock due to rough or prolonged handling of the viscera, and (5) gross soiling of the peritoneal cavity by spillage of the gastrointestinal contents. In addition to observation of sound surgical techniques, the following measures have given successful results. As soon as the operation is completed, the animal is given 750–1500 cc of physiologic sodium chloride solution subcutaneously. This is repeated the next day. Except in operations involving the neck (i.e., esophagostomy) the saline solution may be introduced readily in the large subcutaneous spaces of the neck. We have found subcutaneous as satisfactory as intravenous administration and less troublesome. The animal is also given penicillin intramuscularly, 20,000–50,000 units every 8 hr for the ensuing 2 days. In most instances abdominal bandaging is not required, although it may become necessary for animals that keep disturbing their incisions or pulling at an abdominal cannula.

Perforation of the mucosal septum between the main stomach and the pouch is not uncommon, particularly in the Thomas type pouch when the septum is made unnecessarily long. This usually results when the sutures approximating the mucosal edges are not placed so as to keep the mucosa inverted into the stomach and pouch lumens. Since adhesions between mucosal surfaces do not form, it is essential that only the submucosa of 1 edge be brought into contact with the submucosa of the corresponding edge. This is achieved most readily by use of a continuous Connell suture which passes only through the submucosa and by careful inversion of free mucosal edges. Rupture of the septum seems to be favored also by use of nonabsorbable suture material, which is eventually extruded into the pouch or main stomach, sometimes with establishment of a fistula between them. We have found that the use of absorbable suture material reduces greatly the incidence of septal perforation. Also, because food with high fiber content, such as most commercial dehydrated dog biscuits, tends to cause septal perforation, we feed ground beef heart or horse meat and bread mixed with milk.

Pouch animals do well only when given special care. Continuous drainage of large amounts of acid juice from the pouch rapidly leads to alkalosis, dehydration and death. The larger the pouch and the greater the volume of gastric juice secreted, the more severe the effects. We have found that animals will live indefinitely if 0.1N HCl in approximately the daily quantity of gastric juice lost from the pouch is returned each day with the food. Overdistension of the pouch by accumulated secretion should be prevented by daily draining of the pouch. Animals with distended gastric pouches lose interest in food and show evidence of discomfort. Intrapouch pressure of some Pavlov dogs is sufficient to rupture the septum. On the other hand, continuous drainage of the gastric contents over the skin usually leads to extensive perifistular skin excoriations. This is readily prevented by applying to the affected area a paste made of aluminum hydroxide gel and kaolin. Several milliliters of liquid aluminum hydroxide placed in the pouch each evening is also helpful.

REFERENCES

1. Armour, J.: Canad. M. A. J. 23: 756, 1930.
2. Babkin, B. P.: Personal communication, 1946.
3. Babkin, B. P.: *Secretory Mechanism of the Digestive Glands* (2d ed.; New York: Paul B. Hoeber, Inc., 1950).
4. Bassov, L. V.: Soc. imper. d. natural., Moscow, Vol. 16, Dec. 17, 1842.
5. Beaumont, W.: *Experiments and Observations on the Gastric Juice and the Physiology of Digestion* (Plattsburg, N. Y.: 1833).
6. Bickel, A., and Katsch, G.: *Chirurgische Technik zur Normalen und Pathologischen Physiologie des Verdauungs Apparates* (Berlin: 1912).
7. Blandlot, N.: *Traité analytique de la digestion considereé particulièrement dans l'homme et dans les animaux vertébrés* (Nancy: 1843).
8. Boldyreff, W. N.: Bull. Battle Creek Sanit. & Hosp. Clin. 20: 206, 1925.
9. Brestkin, M. P., and Savitch, W. W.: *Pavlov Jubilee Volume* (Leningrad: 1924), p. 377.
10. Brunschwig, A., *et al.*: J. Clin. Investigation 18: 415–422, 1939.
11. Cope, O., *et al.*: Arch. Surg. 40: 717–732, 1940.
12. Dragstedt, C. A., and Mullenix, R. B.: J. Lab. & Clin. Med. 16: 591, 1931.
13. Dragstedt, L. R., and Ellis, J. C.: Am. J. Physiol. 93: 407, 1930.
14. Frémont: Bull. de l'acad. de méd. 34: 509, 1895.
15. Friedman, M. H. F.: Unpublished observations, 1940.
16. Friedman, M. H. F.: Am. J. Digest. Dis. 10: 447, 1943.
17. Goldberg, S. L., and Mann, F. C.: Ann. Surg. 94: 953, 1931.
18. Heidenhain, R.: Arch. f. d. ges. Physiol. 18: 169, 1878.
19. Hollander, F., and Jemerin, E. E.: Proc. Soc. Exper. Biol. & Med. 39: 87, 1938.
20. Ivy, A. C., and Farrell, J. I.: Am. J. Physiol. 74: 639, 1925.
21. Jemerin, E. E., and Hollander, F.: Proc. Soc. Exper. Biol. & Med. 38: 139, 1938.
22. Klein, E.: Arch. Surg. 25: 442, 1932.
23. Klemensievicz: Sitzungsber. d. wien. Akad. 1875 (cited by Pavlov, 1898).
24. Komarov, S. A.: Arch. f. d. ges. Physiol., Vol. 212, 1926.
25. Komarov, S. A.: Personal communication, 1950.
26. McCrea, E. D.: J. Anat. 59: 18, 1924.
27. McCrea, E. D.: Brit. J. Surg. 13: 621, 1925.
28. Mann, F. C., and Bollman, J. L.: Ann. Surg. 93: 794, 1931.
29. Neuwelt, F.; Olson, W. H., and Necheles, H.: Proc. Soc. Exper. Biol. & Med. 44: 74, 1940.
30. Pavlov, I. P.: *Die Arbeit der Verdauungsdrusen* (Wiesbaden: 1898).
31. Pavlov, I. P., and Simanovskaia, S.: cited in Pavlov, Die operative Meth-

odik des Studiums der Verdauungsdrüsen, in Tigerstedt: *Handbuch der physiologische Methodik* (Leipzig: 1911), Vol. 2, p. 162.
32. Semionova, G. T.: Arch. Sc. biol. Leningrad 44: 97, 1936 (abst., Ber. u. d. ges. Physiol. 105: 433, 1938).
33. Thomas, J. E.: Proc. Soc. Exper. Biol. & Med. 50: 58, 1942.
34. Watman, R. N., and Nasset, E. S.: Ann. Surg. 131: 406, 1950.
35. Webster, D. R., and Armour, J. C.: Tr. Roy. Soc. Canada 26: 109, 1932.

Methods for Study of External Secretory Function of the Pancreas

J. E. THOMAS, *Jefferson Medical College*

Of the numerous and varied techniques used for study of the external secretory function of the pancreas, only a few basic procedures can be described in detail.

Experimental Animals.—Dogs are most useful for study of pancreatic function, although cats have been used extensively for acute experiments, especially abroad. Cats are seldom used for chronic experiments, although Gamble and McIver (27) reported successful experiments on cats with permanent pancreatic fistulas. The dog, because he is co-operative, is generally preferred for chronic experiments, and large, readily accessible ducts in the dog are an added advantage. Because the rabbit pancreas secretes continuously (34) even with the animal under anesthesia (6), rabbits are preferred for certain types of experiments.

For microscopic observation of the living pancreas, rabbits, frogs and mice are used because the pancreatic tissue is more or less diffuse and spread in a thin layer in the mesentery. It is usually possible to find an area near the edge of a mass of pancreatic tissue where a single layer of acini can be observed by transmitted light and individual cells with nuclei and granules can be studied. Some of the most detailed studies have been made on white mice, which are probably the best animals for this type of work (16, 20, 36, 42).

SURGICAL APPROACH TO THE PANCREAS

Anesthesia.—Any of the commonly used surgical anesthetics is satisfactory if the procedure is purely surgical and no experimental observations are to be made. Morphine should not be used because it causes spasm of the duodenal muscle and makes the duodenum difficult to deal with surgically.

For experiments on the pancreas in anesthetized animals, the anesthetic should be adapted to the type of observation to be made. For experiments on dogs involving use or assay of secretin preparations it is customary to use a barbiturate, generally pentobarbital sodium. In cats, urethane (46, 66) or chloralose (14) has been used. The barbiturates depress the cardiac vagus (32) and, presumably therefore, the peripheral parasympathetics generally, so they are not recommended for experiments involving the secretory nerves. In such experiments Babkin (3) found chloralose satisfactory. In Babkin's experiments the animals were 1st anesthetized with chloroform or ether since chloralose alone may cause excitement or even convulsions. Preliminary anesthesia may be omitted if chloralose is administered in a solution of urethane as recommended by Bazett (7). After trying several combinations of these drugs in different proportions, Thomas and Crider (63) found that 4% chloralose in 20% urethane gave excellent results. The chloralose was dissolved in the urethane solution with the aid of heat and the mixture injected intravenously while still warm in a dose of 2 cc/kg of body weight. If the experiment were prolonged, additional smaller doses were given as needed.

Morphine and the inhalation anesthetics usually give poor results. Morphine interferes with collection of the secretion, probably because it causes contraction of the larger ducts. Inhalation anesthetics always causes great excitement in animals, presumably associated with mass stimulation of the sympathetic nerves and release of epinephrine. After such an episode the pancreas may fail to secrete for a long time.

Babkin (3) found that preliminary section of the splanchnic nerves increased the response of the pancreas to various stimuli, probably because it prevents to some extent the inhibitory effects on the pancreas of the operative trauma. Pavlov (48, 49) recommended section of the spinal cord just below the medulla for the same purpose.

OPERATIVE PROCEDURES

a) Location of abdominal incisions.—For acute experimentation and preparation of a permanent fistula, the incision through the abdominal wall should be so located that the pancreas and its ducts can be adequately dealt with without putting undue traction on the mesenteric attachments of the pylorus and duodenum. For most purposes this requirement rules out use of the traditional midline incision. Exposure of the pancreas through the midline can only be accomplished by putting severe traction on the hepatogastroduodenal ligament, and J. O. Crider and I found that traction on this structure results in prolonged inhibition of the pancreas.

In the dog, if only the large duct is sought, a straight incision through the right lateral abdominal wall is adequate. This should begin near the costal margin 2–4 cm to the right of the lateral border of the right rectus muscle and extend caudalward, parallel to the rectus. An incision that will admit 3 fingers is usually adequate. If the accessory duct is also to be dealt with, the incision should begin near the xiphoid process and skirt the costal margin, severing the right rectus near its attachments to the ribs, and continue into the right lateral abdominal wall as far as necessary. An excellent exposure is obtained by combining the 2 incisions just described into what is called a "hockey-stick" incision in human surgery. A midline incision is recommended for the operation of pancreatectomy in which the tail of the pancreas must be exposed (45).

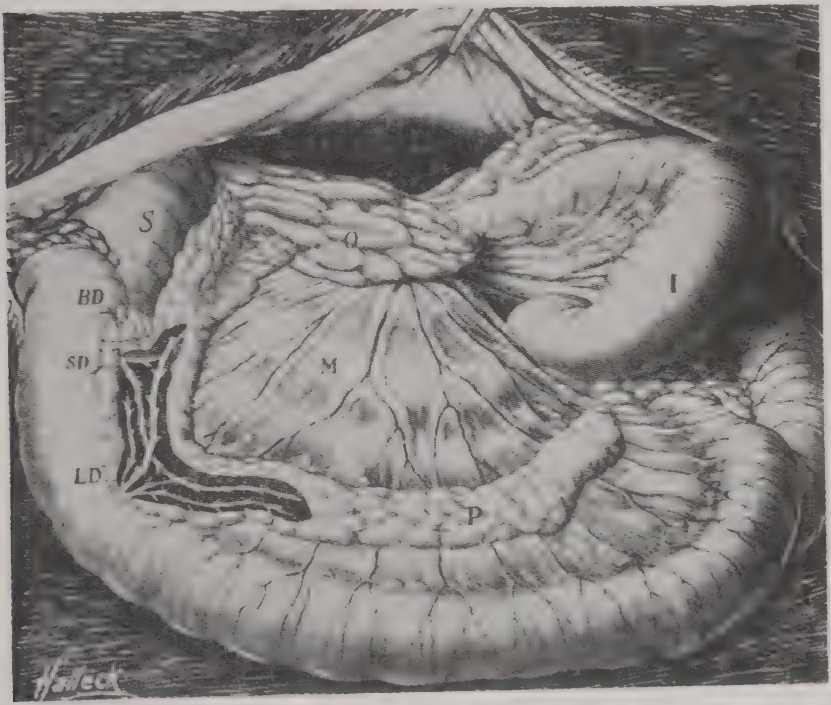

FIG. 1.—Dissection showing position and relations of pancreatic ducts in the dog. *I*, intestine; *O*, omentum; *M*, mesentery; *S*, stomach; *P*, pancreas, uncinate process; *BD*, position of bile duct (dotted, beneath the pancreas); *LD*, large duct and, *SD*, small duct of the pancreas. Pancreas is partly cut away to show position of the ducts. (From Jackson (41), p. 276; a 2d illustration opposite p. 278 will be useful to the beginner.)

b) Exposure and cannulation of pancreatic ducts.—A dissection showing the position and relations of the pancreatic ducts in the dog is illustrated in Figure 1. The corresponding structures in the rabbit and cat are shown in Figure 2. The anatomy of the ducts in the dog has been described in detail by Revell (53). The relations of the pancreatic ducts to the bile duct in several species of laboratory and domestic animals was described by Mann, Foster and Brimhall (44).

The following discussion applies to the dog only. Fingers inserted through an incision made as already described will usually come into immediate contact with the descending portion of the duodenum, which may then be drawn out through the wound with a minimum of traction. Rough handling of the duodenum and traction on the hepatogastroduodenal ligament must be scrupulously avoided. To make sure that all the pancreatic juice is collected through a cannula in the main duct, the accessory duct must be ligated. It is better to locate and tie this duct first because considerable manipulation of the duodenum is required to expose it.

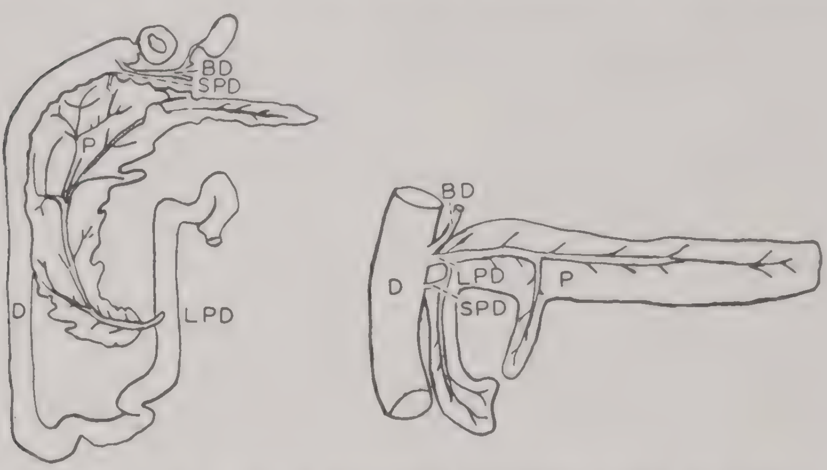

FIG. 2.—Diagrams showing relations of pancreatic ducts, bile ducts and duodenum in the rabbit (*left*) and cat (*right*). *BD*, bile duct; *SPD*, small pancreatic duct; *LPD*, large pancreatic duct; *P*, pancreas; *D*, duodenum. (Modified from Babkin (4) and Greenberg (30); originals by Claude Bernard.)

Comment by Robert Elman

To insure against reconstitution of ducts I have found it necessary to dissect the head of the pancreas completely from the duodenum and tack omentum between them.

To locate the accessory duct, the duodenum is rotated to the left to bring its posterior surface into view. The common bile duct is identified and traced to the point at which it pierces the serous coat of the duodenum. An estimate is made of the location of the biliary papilla, which will be in line with the bile duct and 1 cm or a little more caudad to the point of disappearance of the duct into the duodenal wall. An imaginary horizontal line passing through the biliary papilla at a right angle with the bile duct will parallel the accessory pancreatic duct and frequently lie directly over it. The extramural portion of the duct is covered by pancreatic tissue, but often the intramural portion can be seen as a narrow whitish strand crossing under the longitudinal muscle fibers of the gut, particularly if the duodenum is lightly compressed between thumb and fingers (33). If the duct is not visible it is located by gentle separation of the pancreatic tissue from the duodenum in the area indicated. The remarks in the 2 preceding sentences are equally applicable to the major duct.

After the accessory duct has been disposed of, the duodenum is rotated to the right, i.e., returned to its previous position, and the uncinate process identified. This will be seen adherent to the duodenum along its upper (pyloric) portion but separate and free in the duodenal mesentery lower down. The point of separation is a preliminary guide to location of the large duct which lies between 1 and 1.5 cm (3–4 cm., according to Scott (56)) toward the pylorus from this point. It is usually located by careful observation of the small arteries that run from the pancreas to the duodenum. The duct parallels the 2d or, less often, the 3d of these arteries counting from the point of contact of the pancreas and duodenum (34). The artery that nearly overlies the duct is often larger and more profusely branched than the others; also, the pancreas frequently extends a little farther onto the duodenum directly over the duct than it does elsewhere. The extramural portion of the duct is short and, if a cannula is to be inserted at this time, it is usually necessary to expose the intramural portion, which runs obliquely through the duodenal wall, by dissecting off the duodenal muscle with the point of a knife. Blunt dissection is not used because it requires too much force. After the duct has been cannulated, the body of the cannula may be fastened to the duodenum with a suture to prevent its shifting and consequent kinking of the duct. The rubber drainage tube which will be attached to the cannula should be brought out through the abdominal wall, through a stab wound if necessary, in such position as to maintain alinement of the cannula with the duct when the duodenum is in normal position. The abdominal incision is then closed with sutures even for an acute experiment.

PANCREATIC FISTULAS

Most studies of the external secretion of the pancreas are made by means of a pancreatic fistula through which the pancreatic juice may be collected. These fistulas are temporary, semipermanent or permanent. Temporary fistulas are made by cannulating the duct in an acute experiment. For reasons mentioned previously, they have little value in studies of the effects of normal stimuli, e.g., effects on the pancreas of food in the intestine. Stimuli which act through the central nervous system generally cause no response from the pancreas in the acutely operated, anesthetized animal and even supposedly pure hormonal stimuli such as HCl in the intestine always cause less than a normal response.

The temporary pancreatic fistula is most useful in pharmacologic studies, particularly in study of humoral agents such as secretin. Indeed, it is the method of choice for quantitative studies of secretin activity such as assay of secretin preparations. Assay methods have been described by various workers (14, 25, 28, 31, 40, 61, 66).

Semipermanent pancreatic fistulas.—The first pancreatic fistula, of the semipermanent type, was described by Regner de Graaf in 1664 (17). He cannulated the pancreatic duct of a dog with a quill from a wild duck which he selected "....because that kind of bird hath longer and thinner than all others." The cannula was brought out through the abdominal wall and the pancreatic juice collected in a small vessel attached to its outer end. Claude Bernard (8), Bernstein (10) and others in a similar method used metal tubes. Usefulness was limited because the cannula always came out of the duct in a few days. A part of the preparation for making a temporary or semipermanent pancreatic fistula is illustrated in Figure 3, *A*.

An interesting fistula utilizing the T-tube principle was attributed to Ludwig by Heidenhain (34). Instead of a tube a lead wire was used, looped up in the middle to form a T. The free ends of the wire were inserted into the duct through an incision, 1 end pointing toward the duodenum and the other toward the pancreas. The duodenum was sutured to the abdominal wall and the wire loop forming the stem of the T brought out through the wound, which was closed with sutures. The wound healed leaving a fistulous tract around the wire loop.

In 1923 Rous and McMaster (54) described their technique for semipermanent intubation of the bile duct and in 1927 Elman and McCaughan (22) adapted the method to the pancreas. In this procedure the duct is cannulated outside the intestine and the cannula connected to a long flexible rubber tube, a large part of which is allowed to remain in the peritoneal cavity. The free end is drawn out through a stab wound in the abdominal wall and protected by a sterile collecting bag (Fig. 3, *B*). This fistula is useful for only a short time because of clogging of the cannula, kinking of the tube (57) or bacterial contamination of the juice, resulting in pancreatitis.

A simpler modification was described by Boldyreff and Thompson (12), who inserted a cannula in the duct from within the duodenum and tied it in place with a ligature passed around the extramural portion of the duct. A rubber tube about 8 in. long was attached to the inner end of the cannula and allowed to remain in the intestine. At the same time a permanent, tubulated duodenal fistula was made. To collect the pancreatic juice, the rubber tube was brought out through the fistula by a thread connecting the end of the tube with the fistula. No reports have appeared regarding its successful use by others than the originators. A single at-

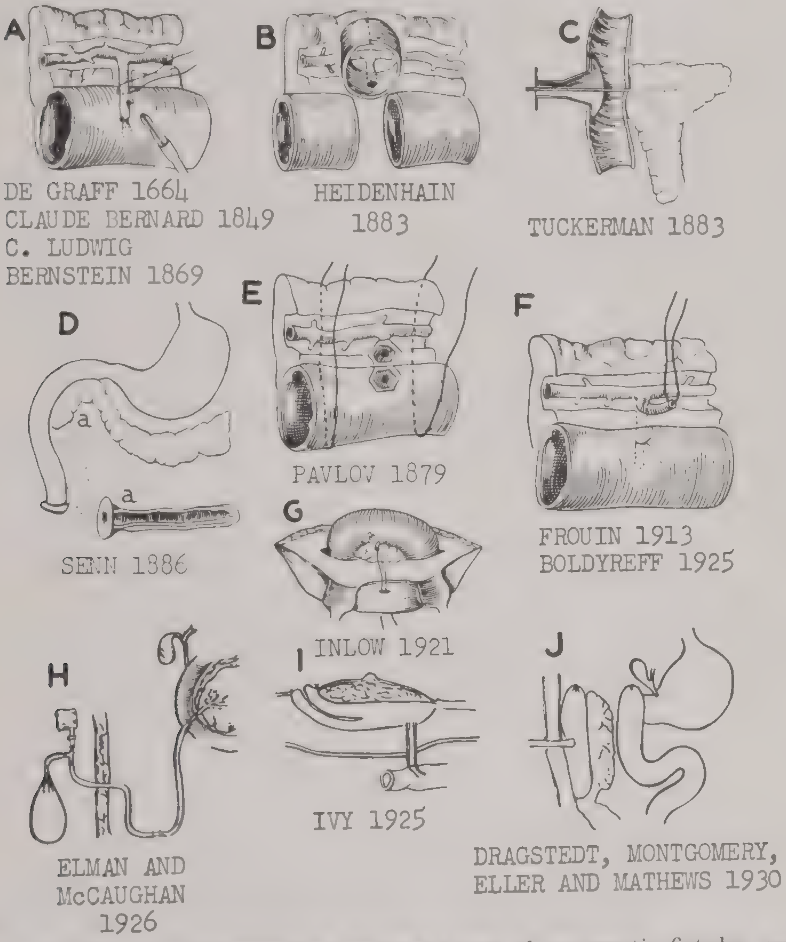
A
B
C
DE GRAFF 1664
CLAUDE BERNARD 1849
C. LUDWIG
BERNSTEIN 1869
HEIDENHAIN
1883
TUCKERMAN 1883
D
E
F
a
a
SENN 1886
PAVLOV 1879
G
FROUIN 1913
BOLDYREFF 1925
INLOW 1921
H
I
J
IVY 1925
ELMAN AND
McCAUGHAN
1926
DRAGSTEDT, MONTGOMERY,
ELLER AND MATHEWS 1930

FIG. 3.—Sketches representing various types of pancreatic fistulas used by different investigators. (Modified from McCaughan (43). Sketches conform to the original except for retouching; only labels have been altered.)

tempt by us to use a method based on similar principles resulted in loss of the cannula in a few days.

Permanent pancreatic fistulas.—Among the earliest successful permanent fistulas of the pancreas is that devised by Pavlov (47, 50). He cut out a rhomboid portion of the duodenal wall surrounding the opening of the major pancreatic duct (Fig. 3, *E*) and, after repairing the duodenum, transplanted the excised papilla, with the duct attached, into the skin of the abdominal wall. The operative technique has been described by Babkin (4) and (in English) by Boldyreff (11). A slightly different operation for the same purpose, proposed independently by Heidenhain (34, p. 179) (Fig. 3, *B*) has no advantage over Pavlov's method. Pavlov's procedure is attended by a high operative mortality (4, 12, 57) and no animal so prepared lives more than a year (12). The trypsin of the juice is activated by contact with the transplanted papilla and causes severe erosion of the skin. Human experience with similar lesions indicates that they cause extreme discomfort and for this reason, if for no other, the method should not be used.

Many objections to Pavlov's fistula were obviated by a modification devised by Babkin (1, 2). After a fistula had been prepared by Pavlov's method, Babkin cut off the exposed mucosa and sutured the duct walls to the margins of the wound. Compression of the duct by scar tissue hindered drainage of the juice to the outside and forced most of it to pass by way of the accessory duct into the intestine. If any juice escaped to the outside it caused no digestion of the skin because it contained no active trypsin. Complete closure of the duct by contraction of the scar was prevented by daily probing, and juice was collected when desired through a glass cannula inserted in the duct. Babkin (4) reported that animals so prepared lived for 3–4 years in good health.

Direct transplantation of the duct into the skin without the papilla (Fig. 3, *F*) has been proposed from time to time (11, 26). The most successful technique for this purpose was devised by Inlow (39) (Fig. 3, *G*). He implanted the portion of the intact duodenum containing the pancreatic papilla into the subcutaneous tissue of the abdominal wall. At the same time or, preferably, later, the intramural portion of the duct was dissected and cut near the papilla and the free end brought out through a stab wound in the skin made directly over the base of the duct. In the original operation a catheter was inserted in the duct to facilitate handling and left in place until it was expelled spontaneously. Scott (56) prefers to suture the duct directly to the skin. This fistula has the advantages of Babkin's modification of the Pavlov fistula and is less difficult to prepare. Details of the operative technique were given by Inlow (39) and, more fully, by Scott (56).

Dragstedt *et al.* (19) perfected a pancreatic fistula (Fig. 3, *J*) which, like that of Pavlov, yields pancreatic juice containing active trypsin. They isolated a portion of the upper duodenum, including the biliary and pancreatic papillae, by separating it from the stomach and lower duodenum. The pyloric portion of the stomach was excised and the lower duodenum anastomosed to the gastric stump. The common bile duct was transplanted into the stomach or lower duodenum. The isolated segment of duodenum, which included the pancreatic papillae, was closed at both ends and a gold-plated metal cannula inserted through its wall and brought to the outside through the abdominal wall to provide drainage for the mixed pancreatic and duodenal secretions. One advantage claimed for this method over the Pavlov fistula is that the juice, draining through a cannula, does not spread over the skin and cause irritation and erosion.* An obvious disadvantage is that the extensive surgery in the vicinity of the pylorus is almost certain to result in the severing of some of the important nerve fibers that supply the pancreas.

The foregoing criticism does not apply to a modification of this fistula suggested by Dragstedt and described in detail by Scott (56). In this procedure only the major duct is included in the isolated segment of duodenum and the continuity of the gut is reestablished by end-to-end anastomosis. This modification has the added advantages that some of the juice drains into the intestine by way of the accessory duct and the bile duct is not disturbed; consequently digestion proceeds normally and the severe systemic disturbances associated with complete loss of pancreatic juice are considerably diminished.

Comment by L. R. Dragstedt

This method has been used extensively in our laboratory with success. If complete diversion of all pancreatic secretion from the intestines is desired it is advisable to remove the tail and part of the body of the pancreas, leaving only that part in the duodenal mesentery and adjacent to the lower duct. This partial pancreatectomy also decreases the amount of pancreatic juice lost and resultant demineralization and acidosis are easier to control. When it is desired to make certain that no pancreatic juice is discharged into the intestine, the portion of the pancreas in the neighborhood of the lesser duct should be removed and a layer of omentum inserted.

---

* A modification of this fistula proposed by Baronofsky (Surgery 23: 665, 1948) allows for drainage of the pancreatic juice into the intestine in the intervals between observations.

An ingenious and original method of preparing a permanent fistula which yields pancreatic juice that contains trypsinogen instead of active trypsin was described by McCaughan (43). His method is based on the demonstrated facts that the pancreatic ducts anastomose freely in the gland and the juice may be made to flow in either direction. Both pancreatic ducts in a dog were exposed and cut between ligatures. The uncinate process was freed from its mesenteric attachments and its lower end brought out through a wound in the abdominal wall and secured to the wall by sutures. The exposed end was then amputated and, after healing, the juice was collected through a cannula inserted in the severed duct of the uncinate process. Several steps in the operation are illustrated in Figure 4. McCaughan reported no dilatation of the ducts discernible at autopsy and no atrophy of acinous tissue such as might be expected if the drainage were not adequate. He credits Senn (59) with a similar technique, but Senn's fistula (Fig. 3, *D*) drained only a portion of the pancreas and resulted in ultimate failure of secretion owing to atrophy of the gland.

Care of pancreatic fistula animals.—An animal with a total fistula of the pancreas suffers from disturbances of digestion and absorption, water and electrolyte balance and from local irritative lesions. (For literature see (4) and (61).) Digestion is usually normal in animals with incomplete fistulas, but even in these the other disturbances mentioned occur; the severity depends on the amount of pancreatic juice lost.

The alimentary defect chiefly affects digestion and absorption of fat and protein. Ideal treatment for this would seem to be return of the pancreatic juice directly into the intestine, but this is not always feasible and seems to have little advantage over other methods of returning the juice (15). The juice may be given with the food, or whole pancreas or preparations of pancreatic enzymes may be administered (55). According to Coffey *et al.* (15), none of these measures wholly corrects the deficiency. The food provided should be easily digestible and cause minimal stimulation of the pancreas. A diet of bread and milk (50) or dog biscuit and milk (56) has been recommended.

Total loss of pancreatic juice causes death in 5–8 days with dehydration and decrease in total base of the blood (18, 22, 27). Loss is apt to be aggravated by hypersecretion of the pancreas (4, 9, 34) which appears to be associated with inadequate neutralization of the gastric juice; gastric hypersecretion may also be a factor (65). The acid of the gastric juice acts through the secretin mechanism to increase the flow of pancreatic juice. Treatment consists of administration with the food or drinking water of pancreatic juice, sodium bicarbonate or sodium chloride. The salts may also be administered parenterally. Scott (56) recommends sodium chloride in a dose of 0.5 g/kg body weight/day. Addition of potassium and calcium salts in the proportions found in the juice (approximately those of the blood) seems rational; Elman and Hartman (21) and Dragstedt (18) used Ringer's solution intraperitoneally or intravenously. The dose used by the former was 500 cc/animal daily. Even with the best available therapy, survival time of animals with total fistula is limited, and that of animals with in-

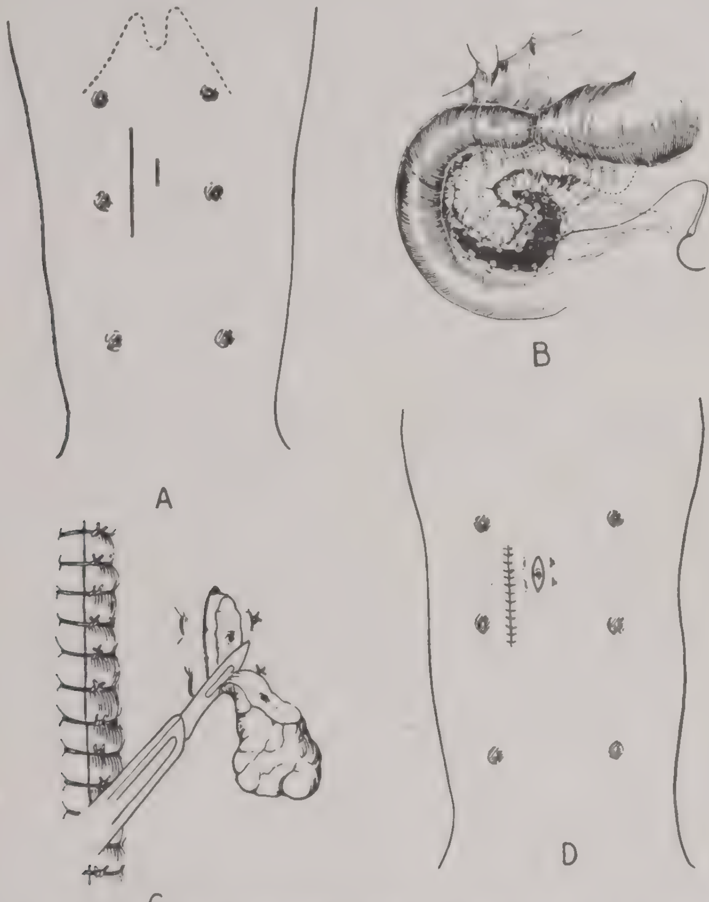

FIG. 4.—Technique of the retrograde external pancreatic fistula according to McCaughan. *A*, incisions. *B*, the 2 pancreatic ducts are divided between ligatures and the uncinate process freed from its attachments. *C*, uncinate process is drawn through the stab incision and anchored to the wound with mattress sutures, avoiding constriction of the duct. The tip of the process is amputated with a scalpel about 1 cm above the level of the skin and the abdominal wound closed in layers. *D*, final appearance of fistula preparation. (From McCaughan (43).)

complete fistulas is inversely related to the amount of juice lost (57).

Local irritative lesions are due to contact of pancreatic juice with the skin. If active trypsin is present, as in the Pavlov or Dragstedt fistula, there may be actual digestion of tissue, but even inactive juice may cause dermatitis, apparently owing to its alkaline reaction (58). The remedy is to prevent contact of the juice with the skin, as in Dragstedt's method. Lesions are treated by liberal application of an aluminum hydroxide paste such as Protogel (24).

COLLECTION OF PANCREATIC JUICE WITHOUT PANCREATIC FISTULA

It is evident that the best designed pancreatic fistulas are difficult to prepare and troublesome to maintain. Furthermore, they often cause abnormality of the pancreatic secretion (34) and so may yield incorrect or even misleading experimental results. These facts prompted Thomas and Crider (62) to devise a method for collecting pancreatic juice through a permanent duodenal fistula, leaving the pancreas and its ducts in their normal relations to the duodenum and to their nerve and blood supply. A similar procedure was attempted by Tuckerman (64) in 1883 (Fig. 3, *C*) but without having notable success.

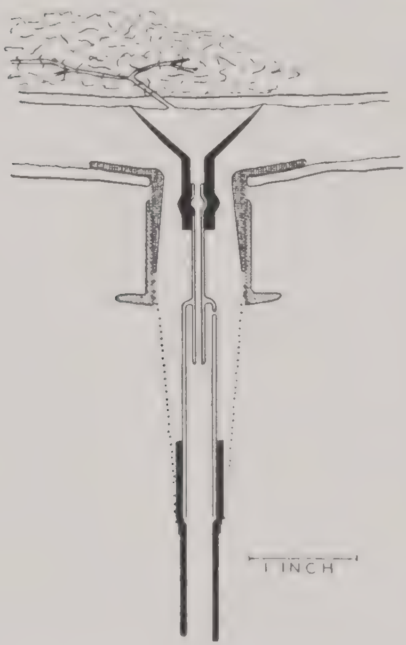

Fig. 5.—Duodenal fistula used for collecting pancreatic juice. In the present technique a glass cannula (Fig. 6) in the pancreatic duct is substituted for the funnel shown here. (From Thomas and Crider (62).)

In the experiments of Thomas and Crider a duodenal fistula was prepared and fitted with a fairly large (minimum diameter, $^5/_8$ in.) fistula tube (60) so placed as to be directly opposite the opening of the major pancreatic duct. By ligating the accessory duct while preparing the duodenal fistula, all the pancreatic juice was made to flow through the main duct. In their early experiments they collected the juice through a soft rubber funnel inserted through the fistula tube and pressed against the mucosa surrounding the pancreatic papilla. The method is illustrated in Figure 5. Pancreatic secretion collected thus is similar to that obtained with the original Pavlov fistula in that it is mixed with a small amount of duodenal secretion and therefore contains active trypsin. Scott and his co-workers (58) modified the procedure by cannulating the pancreatic duct through the duodenal fistula and obtained pure pancreatic juice in which trypsinogen was not activated. About the same time Quigley (51a) independently developed the method of cannulating the duodenum with a Monel metal cannula placed opposite the major pancreatic duct and collecting juice from a glass cannula introduced through the duodenal cannula and into the pancreatic duct.

Preparation of a suitable duodenal fistula is not difficult if an appropriate fistula tube, e.g., one of the type described by Thomas (60), is used, although some workers complain of difficulty in cannulating the duct. The procedure as outlined by Scott in conversations with Thomas and Crider and subsequently practiced by them and their associates is described in some detail.

PROCEDURE

The animal is supported in a standing position on a laboratory table by a muslin harness suspended from a $^3/_4$ in. horizontal rod supported by upright rods firmly secured to the ends of the table. A good light is required which may be held in the hand or reflected into the fistula tube by a head-mirror. Bent glass probes and cannulas with tips of various sizes, from 1–4 mm in diameter, are required. The cannulas may be made of metal (58) or of glass (Fig. 6).

After the papilla is clearly seen the smallest probe is inserted in the duct. This is usually accomplished without difficulty and serves to indicate the exact position and angle of the duct. From the papilla the duct always courses toward the head and usually slightly upward, i.e., toward the back, and of course medialward. The first probe is withdrawn and larger sizes are tried in succession until one is found that is too large to enter the duct. A cannula is chosen corresponding in size to the largest probe that will enter the duct. Usually it may be inserted without difficulty. If resistance is encountered, one should make sure that the tip of the cannula is in the duct orifice and then with very gentle pressure, continued for perhaps a minute or more, force it past the constriction in the duct, bearing in mind that the resistance is due to contraction of smooth muscle, which reacts to vigorous stretching force with further contraction but will yield in time to gentle pressure. An immediate flow of juice through the cannula indicates proper positioning in the duct. If several attempts to insert the cannula fail and the duct or surrounding duodenal tissue has been traumatized, it is best to abandon the procedure and make another attempt in about 30 min, allowing time for relaxation of the ducts and, perhaps, the investigator.

Once the cannula is in place a rubber collecting tube of about 1 mm bore is attached to its distal end and pinned to the muslin harness in such position as to support the cannula weight and relieve it of all strain except such as will tend to maintain it in proper position.

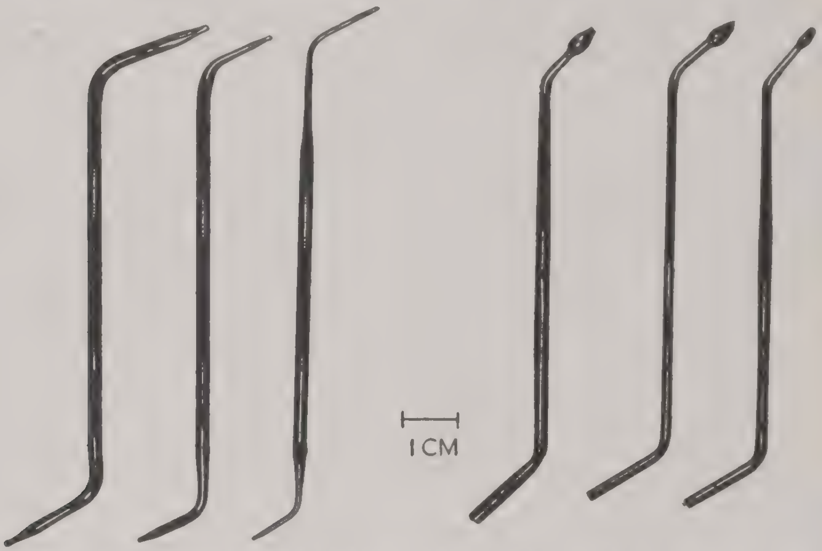

FIG. 6.—Glass probes (*left*) and cannulas (*right*) used in cannulating the pancreatic duct through a duodenal fistula. The bend at the outer (lower) end of the cannulas is essential for ease of handling. The articles were coated with India ink before being photographed.

The first cannulation of any 1 duct is the most difficult. Subsequent cannulations become progressively easier and can usually be done without the initial probing. If the duct is not cannulated for several weeks or months, difficulty may again be encountered. Once the technique of cannulating the duct is mastered the pancreas becomes, instead of the most difficult, one of the easiest of the digestive glands to investigate. If a gastric fistula is made at the same time as the duodenal fistula—the invariable practice in our laboratory—many problems involving the pancreas can be investigated since there is access not only to the pancreatic duct but also to the stomach and duodenum for administration of food or other substances that may influence pancreatic function. The animals require no special care and remain in good health indefinitely. A disadvantage is that the animals must be under observation while the juice is being collected. This difficulty may eventually be overcome, but until it is the method is not applicable to problems requiring collection of the secretion over long periods.

EXPERIMENTAL PANCREATIC INSUFFICIENCY

The functions of the external pancreatic secretion may be inferred from the results of excluding pancreatic juice from the intestine. This may be accomplished by establishment of a complete pancreatic fistula, by removal of the pancreas or by obstruction to the flow of pancreatic juice. Fistulas of the pancreas have been discussed. The technique of pancreatectomy is outside the scope of this discussion; a satisfactory description of the operation performed on dogs has been given by Markowitz (45).

Flow of pancreatic juice into the intestine, with the pancreas in situ, may be prevented by ligation or avulsion of the pancreatic ducts. Simple ligation of the main and accessory ducts is generally ineffective. The ligature may cut through the duct wall, allowing the duct to become re-established, or, if the ligature holds, a cyst may form on the pancreatic side of the obstruction and rupture into the intestine (51). The probable presence of supernumerary ducts must also be considered (13, 35). According to Bottin (13), a 3d duct enters the duodenum midway between the 2 larger ducts in 20–25% of dogs. It has been repeatedly demonstrated that only a small amount of pancreatic tissue in communication with the duodenum is needed to prevent symptoms of pancreatic insufficiency.

To be effective, the ducts must be so ligated as to maintain complete separation of the pancreas from the duodenum. This may be accomplished by the method of F. T. Murphy, described by Pratt *et al.* (51). All pancreatic tissue is carefully dissected away from the duodenum and the visible ducts and the smaller vessels that bind the pancreas to the duodenum are tied and cut, with care to preserve an adequate blood supply to the duodenum. The omentum is then drawn up between pancreas and duodenum and wrapped at least part way around the duodenum. In this way a permanent barrier is established between the duodenal and pancreatic ends of the severed ducts.

Avulsion of the ducts was used successfully by Hoerner (37) in Mann's laboratory. Hoerner's description of the technique follows: "Each duct was exposed at its point of entrance into the duodenal wall. A small hemostat was placed securely on each duct and the duct was sectioned between the clamp and the duodenal wall. The clamp was rotated, winding the duct around its lower end. In this manner all the ductal system was removed from the gland except the smaller tributaries." No mention was made of anomalous ducts or how they were dealt with. Probably the larger ones would be avulsed along with the major ducts, but separation of the pancreas from the duodenum in the area between the main and accessory ducts would provide added assurance that the smallest ducts were severed. Symptoms of pancreatic insufficiency may be expected a few days after pancreatectomy, establishment of a complete fistula or effective ligation of the pancreatic ducts.

SPECIAL TECHNIQUES

Numerous other methods applicable to special problems have been used. Farrell and Ivy (23) (Fig. 3, *I*) transplanted a portion of the pancreas into the mammary gland of a lactating bitch and studied the secretion after severing the original blood and nerve supply and thus obtaining a completely denervated portion of pancreas. Houssay and Molinelli (38) transplanted the pancreas and duodenum into the neck in acute experiments by anastomosing the blood vessels. Babkin and Starling (5) and Goldstein (29) have described methods for perfusing the excised pancreas. Ramsay, Thomas and Crider (52) have described a method for exteriorizing a portion of the pancreas. They used it to obtain biopsy specimens for study of histologic changes during secretion, but it should also be applicable to studies of the enzyme content of pancreatic tissue at different stages of digestion or restitution of the gland following secretory activity.

REFERENCES

1. Babkin, B. P.: Tr. Imper. Mil. M. Acad., St. Petersburg 9: 113, 1904 (Russian). (Cited by Babkin (4).)
2. Babkin, B. P.: in Tigerstedt, *Handbuch der physiologische Methodik* (Leipzig: 1908), Vol. II, pt. 2, p. 117.
3. Babkin, B. P.: J. Physiol. 59: 153, 1924.
4. Babkin, B. P.: *Die aussere Sekretion der Verdauungsdrüsen* (2d ed.; Berlin: Julius Springer, 1928).
5. Babkin, B. P., and Starling, E. H.: J. Physiol. 61: 245, 1926.
6. Baxter, S. G.: Am. J. Physiol. 96: 343, 1931.
7. Bazett, H. C.: Personal communication.
8. Bernard, C.: Arch. gen. d. med. 1: 60, 1849.
9. Bernard, C.: *Liquides de l'organisme* (Paris: 1859), Vol. II.
10. Bernstein, N. O.: *Zur Physiologie der Bauchspeichelabsonderung* (Leipzig: 1869). (Cited by Babkin (4).)
11. Boldyreff, W. N.: Bull. Battle Creek Sanit. & Hosp. Clin. 20: 206, 1925.
12. Boldyreff, W. N., and Thompson, O. E.: Tohoku J. Exper. Med. 38: 581, 1940; Am. J. Physiol. 129: P. 313, 1940.
13. Bottin, J.: Compt. rend. Soc. de biol. 117: 825, 1934.

14. Burn, J. H., and Holton, P.: J. Physiol. 107: 449, 1948.
15. Coffey, R. J.; Mann, F. C., and Bollman, J. L.: Am. J. Digest. Dis. 7: 144 and 149, 1940.
16. Covell, W. P.: Anat. Rec. 40: 213, 1928.
17. de Graaf, R. (1664): English translation quoted by Fulton, J. F.: *Selected Readings in the History of Physiology* (Springfield, Ill.: Charles C Thomas, Publisher, 1930).
18. Dragstedt, L. R.: Am. J. Surg. 11: 544, 1931.
19. Dragstedt, L. R.; Montgomery, M. L., and Ellis, J. C.: Proc. Soc. Exper. Biol. & Med. 28: 109, 1930.
20. Duthie, E. S.: Proc. Roy. Soc. London, SB 114: 20, 1933.
21. Elman, R., and Hartman, A. F.: Arch. Surg. 23: 1030, 1931.
22. Elman, R., and McCaughan, J. M.: J. Exper. Med. 45: 561, 1927.
23. Farrell, J. I., and Ivy, A. C.: Am. J. Physiol. 77: 474, 1926.
24. Friedman, M. H. F.: J.A.M.A. 131: 520, 1946.
25. Friedman, M. H. F., and Thomas, J. E.: J. Lab. & Clin. Med., in press.
26. Frouin, A.: Compt. rend. Soc. de biol. 74: 1283, 1913.
27. Gamble, J. L., and McIver, M. A.: J. Exper. Med. 48: 849, 1928.
28. Gershbein, L. L.; Wang, C. C., and Ivy, A. C.: Proc. Soc. Exper. Biol. & Med. 70: 516, 1949.
29. Goldstein, B.: Ztschr. f. d. ges. exper. Med. 75: 182, 1931.
30. Greenberg, J.: Yale J. Biol. & Med. 6: 121, 1933.
31. Greengard, H., and Ivy, A. C.: Am. J. Physiol. 124: 427, 1938.
32. Gruber, C. M.; Gruber, C. M., Jr., and Colosi, N. A.: J. Pharmacol. & Exper. Therap. 63: 215, 1938.
33. Heidenhain, R.: Arch. f. d. ges. Physiol. 10: 557, 1875.
34. Heidenhain, R.: in Hermann: *Handbuch der Physiologie* (Leipzig: 1883), Vol. 5, p. 1.
35. Hess, O.: Arch. f. d. ges. Physiol. 118: 536, 1907.
36. Hirsch, G. C.: Ztschr. f. wissenschaft. Biol. (Ztschr. f. Zellforsch. u. mikr. Anat.) 15: 37 and 290, 1932.
37. Hoerner, M. T.: Am. J. Digest. Dis. 2: 295, 1935.
38. Houssay, B. A., and Molinelli, E. A.: Compt. rend. Soc. de biol. 97: 1032, 1927.
39. Inlow, W. DeP.: J. Lab. & Clin. Med. 7: 86, 1921.
40. Ivy, A. C., *et al.*: Am. J. Physiol. 95: 37, 1930.
41. Jackson, D. E.: *Experimental Pharmacology* (St. Louis: C. V. Mosby Company, 1917).
42. Kühne, W., and Lea, A. S.: Untersuch. a. d. physiol. Inst. d. Univ. Heidelberg 2: 448, 1882.
43. McCaughan, J. M.: Am. J. Digest. Dis. 6: 392, 1939.
44. Mann, F. C.; Foster, J. P., and Brimhall, S. D.: J. Lab. Clin. & Med. 5: 203, 1920.
45. Markowitz, J.: *Experimental Surgery* (2d ed.; Baltimore: Williams & Wilkins Company, 1949).
46. Mellanby, J.: J. Physiol. 61: 419, 1926.
47. Pavlov, I. P.: Verhandl. d. XI Naturforschervereinigung, St. Petersburg 11: 51, 1879. (Cited by Babkin (4).)
48. Pavlov, I. P.: Centralbl. f. Physiol. 2: 137 and 138, 1888.
49. Pavlov, I. P.: Arch. f. d. ges. Physiol., Supp., 1893, p. 176.
50. Pavlov, I. P.: *The Work of the Digestive Glands;* tr. by W. H. Thompson (2d ed.; Philadelphia: J. B. Lippincott Company, 1910).
51. Pratt, J. H.; Lamson, P. D., and Marks, H. K.: Tr. A. Am. Physicians 24: 266, 1909.

51a. Quigley, J. P.: Personal communication.
52. Ramsay, A. J.; Thomas, J. E., and Crider, J. O.: Anat. Rec. 86: 87, 1943
53. Revell, D. G.: Am. J. Anat. 1: 443, 1902.
54. Rous, P., and McMaster, P. D.: J. Exper. Med. 37: 11, 1923.
55. Schmidt, C. R., *et al.:* J. Nutrition 14: 513, 1937.
56. Scott, V. B.: J. Lab. & Clin. Med. 25: 1215, 1940.
57. Scott, V. B.: Personal communication, 1948.
58. Scott, V. B., *et al.:* Am. J. Physiol. 134: 208, 1941.
59. Senn, N.: Am. J. M. Sc. 92: 141, 1886.
60. Thomas, J. E.: Proc. Soc. Exper. Biol. & Med. 46: 260, 1941.
61. Thomas, J. E.: *External Secretion of the Pancreas* (Springfield, Ill.: Charles C Thomas, Publisher, 1950).
62. Thomas, J. E., and Crider, J. O.: Am. J. Physiol. 131: 349, 1940.
63. Thomas, J. E., and Crider, J. O.: J. Pharmacol. Exper. & Therap. 87: 81, 1946.
64. Tuckerman, L. B.: J.A.M.A. 1: 172, 1883.
65. Walther, A. A.: Dissert., St. Petersburg, 1897. (Cited by Babkin (4).)
66. Wilander, O., and Agren, G.: Biochem. Ztschr. 250: 489, 1932.

Insulin-Acidity Test for Determining Integrity of Vagal Influence on the Stomach

FRANKLIN HOLLANDER, *Mount Sinai Hospital, New York*

Interest in vagotomy as surgical treatment for peptic ulcer, popularized in this country by Dragstedt, made a physiologic test of the functional continuity of the gastric vagi in man an urgent requirement. The diversity of clinical results which followed the operation contributed greatly to the need for a completely objective test for the presence or absence of vagal control in the stomach. The need became increasingly evident with publication of anatomic observations on man regarding the extensive ramifications of the esophagogastric vagi. Furthermore, these structural ramifications vary greatly from individual to individual. In addition to the clinical importance of a vagal test, physiologists have found it necessary for many years to use such a procedure for clear differentiation of Pavlov and Heidenhain pouches, particularly when a Pavlov pouch preparation fails to give the expected reactions.

Any acceptable evidence of vagal integrity must be based on a physiologic reaction in response to a well defined stimulus rather than on morphologic study. Furthermore, the vagi must be stimulated at or above the cervical region, preferably at the vagus center, so that the entire length of the esophagogastric portion of both nerves is subjected to the stimulus. The response, preferably in a quantitative form, must be observed as a reaction of the stomach. Thus, various modes of stimulation have been investigated or are available a priori: (*a*) physical agents, i.e., electricity; (*b*) psychic stimuli, i.e., sight, smell or taste of food (8, 11, 12, 21, 24); (*c*) centrally acting pharmacologic agents, e.g., amino acids (19, 25); chloralose-urethane (18), ethyl 3:3 dimethylallyl barbituric acid (2, 16), and insulin in dosage sufficient to induce considerable hypoglycemia (for early references, see Jemerin *et al.* (12)). The end-organ responses which have merited consideration are: (*a*) spontaneous hunger contractions which are augmented by insulin in the normal subject but are inhibited by insulin following vagotomy (17); (*b*) gastric tone and type II and III gastric contractions which normally are augmented by insulin hypoglycemia but are depressed by insulin after vagotomy (10, 17, 20); (*c*) the volume of gastric juice, its pepsin content or its concentration of mucoprotein (3) or its concentration of free HCl (12), each of which is increased by adequate stimulation of the intact vagi but is not augmented if *all* the vagal pathways to the stomach have been interrupted.

Only one combination of these modes of central nerve stimulation and end-organ reactions has been formulated as a definite test procedure, having a definitive empiric characterization of the response with or without intact functioning vagal innervation. The procedure is based on the production of insulin-induced hypoglycemia of sufficient magnitude to initiate or augment HCl secretion in the stomach. This hypoglycemic state, and not the insulin per se, stimulates the vagus centrally, and impulses are carried to the end-organ via the vagal trunks (1).

Of the remaining stimulatory processes and gastric reactions, output of mucoprotein in response to insulin hypoglycemia has also been presented as a completely formulated test procedure. However, the extent to which the gastric mucous glands are directly stimulated by the parasympathetic nerves is not yet established; certainly part of this neural control has already been shown to operate indirectly through mechanical stimulation of the gastric musculature (6, 9), and there is some evidence that this may be true also for the remainder. Hence, this test for vagal integrity cannot be accepted as a standard method until it has been clarified and has received independent confirmation by other investigators. Many of the other possibilities have been shown specifically to be inadequate for various reasons (23). Accordingly, the only test generally accepted is the insulin-acidity test, the procedure (7) for which is presented here.

PROCEDURE

1. The patient is given a light diet the day before the test, with complete deprivation of food after 7:00 P.M. and of fluid after midnight.

2. As early as convenient on the morning of the test, a 14 F. Levin tube is passed by mouth, with the usual sips of water omitted. The tube must not be removed during the test period. The patient should expectorate all saliva.

3. The fasting contents are aspirated completely with a 50 ml Luer syringe (specimen "FC"), then 2 successive samples of resting secretion are aspirated at 15 min intervals. Immediately after the second resting sample is taken, a sample of blood is drawn for the fasting blood glucose determination.

4. Twenty units of unmodified ("regular") insulin is injected intravenously and the time of injection recorded. Sterile glucose solution should always be available for intravenous administration in case symptoms of severe hypoglycemia develop. There is no systematic evidence that subcutaneous or other mode of administration is suitable, and until rigorously tested evidence on this point is adduced the intravenous procedure should be used.

5. Every 15 min after the injection the stomach contents are completely aspirated, and a record is made of the volume and time of collection and of presence of mucus and bile. A sample is kept for acidity titrations. Immediately following every second such aspiration except the last (i.e., every 30 min after the injection), a sample of venous blood is drawn for glucose determination. The aspirations are continued for 2 hr, thus giving 11 gastric samples, including the "FC" and resting specimens, and 4 blood samples, including the fasting control.

6. Analytic methods. Free and total acidities are determined by any reliable method; for microtitration of 0.5 ml samples the procedure of Hollander (5) is recommended because of its precision. This entails use of buffer standards containing indicator for the precise determination of the end-points: pH 3.5 with bromphenol blue for free acidity, and pH 7.0 with phenol red for total acidity. Glucose is determined on whole blood by the Shaffer-Hartmann-Somogyi method using the blood filtrate following tungstic acid precipitation, the specimens being mixed with sodium fluoride and thymol to prevent autoglycolysis as well as coagulation. For investigative purposes, all determinations are done in duplicate, and the mean acidity and blood sugar data are plotted against the collection time in the usual way.

Conditions to be met to assure validity.—For the test to be valid, the following conditions must obtain; otherwise the results must be discarded and the test repeated.

1. There must be suitable evaluation of the "spontaneous" resting secretion for control purposes. These control specimens must be taken immediately before insulin injection. The acidity of a "fasting" specimen which has been retained in the stomach for hours before aspiration is not a valid control.

2. The stomach must be properly intubated. As a rule, complete absence of secretion during the 30 min of the control period, or aspiration of specimens which resemble duodenal contents (containing large amounts of bile) in cases in which extensive intestinal regurgitation is not to be expected, may be taken as evidence of improper positioning of the stomach tube.

3. The blood sugar curve must reflect adequate hypoglycemia, defined empirically by a blood sugar level at least as low as 50 mg/100 ml. If the Somogyi $Zn(OH)_2$ method be used for precipitating the blood proteins, the measure of adequate hypoglycemia must be taken at a lower level, about 45 mg/100 ml. A minimal value slightly above this critical level is acceptable only if the test result is unequivocally positive, and especially if the acidity curve begins to rise at the time corresponding to attainment of the lowest blood sugar value. The true minimum often will have been attained at a time *between* successive samples and therefore will have a value somewhat less than the lowest concentration actually observed. A single blood sugar determination, made on a specimen taken 30 min after insulin injection, cannot be substituted for a complete curve based on at least 3 such determinations. This was shown by a series of 79 successful insulin tests, 55 (70%) of which showed a minimum blood sugar value at the end of the 1st 30 min period and the rest at the end of the second or third half-hour. In the latter circumstances, dependence on a single 30 min blood specimen would have necessitated repetition of $^1/_3$ of all the tests because of supposedly inadequate hypoglycemia. Likewise, the insulin dosage alone, without blood sugar determinations or such physical signs as perspiration, vertigo and diplopia, is only confirmatory and cannot be used as evidence of adequate hypoglycemia in place of the blood sugar curve. All are unreliable because of the poor correlation between their time of onset and intensity in different individuals and the degree of hypoglycemia. When the degree of hypoglycemia is inadequate, repetition of the test with a slightly larger dose of insulin may be advisable. On the other hand, it has been suggested that an excessive degree of hypoglycemia may have less stimulating effect than a moderate degree, but this awaits confirmation as a generalization.

4. When all gastric specimens obtained during the insulin-acidity test fail to reveal any free acid (i.e., the pH of all is greater than 3.5), it must be demonstrated that the subject is capable of secreting a recognizable amount of HCl at the time of the test. In the absence of other evidence that the parietal cells have not completely lost their ability to form acid, temporarily or otherwise, a test of the secretory responsiveness to histamine (at least 0.01 mg/kg body weight) should be performed immediately after cessation of the insulin test.

Interpretation and evaluation of results.—The acidity responses which are valid according to the criteria just given are classified as positive, negative or equivocal.

In a POSITIVE TEST the free acidity curve manifests a well defined rise following injection of the insulin. Such a rise has been characterized arbitrarily by the presence on the curve of 2 or more successive points which lie distinctly above the control level established by the mean of the resting specimens. At least 1 of these points must be elevated by 20 or more units (mEq/1), unless the resting specimens contain no free acid whatever, in which case a rise of 10 mN is accepted as significant. This definition is based on the presumption that the acidity titration technique employed in the test is reliable to ±3 mN or better, which is not invariably the case with titration procedures used routinely in gastric analysis. Unfortunately, the absolute height of the acidity curve, duration of the elevated portion and latent period between the time of injection and the beginning of the rise depend on so many variables that no definitive conclusions can at present be drawn in relation to the objectives of this test.

In a true NEGATIVE TEST the acidity curve either shows a downward slope without a subsequent rise or else is flat by the aforementioned criteria. A flat curve resulting from failure of the stomach to secrete any free acid whatever is negative only if the ability of the stomach to secrete acid is demonstrated independently on the same day.

An EQUIVOCAL TEST is one in which the acidity curve does not clearly conform to the criteria for either a positive or a negative response. Sometimes the curve fluctuates above and below the control value because of inadequate emptying of the fasting contents from a hypotonic stomach or because the glands are operating at a low level of secretory activity in general—as a result of which a relatively low output of acid may be reduced intermittently by intragastric neutralization. An equivocal test should be repeated, with special care to evacuate the stomach completely before the test is started.

A positive reaction unequivocally indicates functional as well as morphologic continuity of some secretory nerves to the stomach. These may be either truly vagal or vagomimetic. Inasmuch as the test is qualitative, even though it is based on the quantitative determination of gastric acidity and blood sugar concentration, it affords no quantitative measure of the degree of vagal continuity or activity present, either before or after vagotomy. Evaluation of the degree of vagal continuity would be highly important for both clinical and experimental purposes, but no method for such quantification has been proposed. This need is felt particularly in cases of incomplete total vagotomy which occur despite complete transection of the main trunks of the gastric vagi.

When a positive result is obtained following an apparent bilateral vagotomy, it is generally believed that the neural impulses induced centrally by the insulin hypoglycemia are transported to the stomach in one of several ways: (*a*) by branches of the vagus missed at operation, because they leave the main nerves well above the diaphragm and pass through it to the stomach at some distance from the main (subdiaphragmatic) gastric vagus trunks; (*b*) by vagal branches buried in the esophageal and gastric walls, either as distinct nerve elements or after fusion with the intramural nerve plexus, or (*c*) by way of cholinergic nerves which arise in the thoracic segments of the spinal cord and pass to the stomach through the celiac plexus, such as have been shown to transport motor impulses in the dog (4, 13–15) and which may exercise secretory influences as well (22). Dogs with partially innervated Pavlov pouches, as well as the more completely innervated Hollander-Jemerin pouches (8, 11, 12), also give a positive reaction.

The implications of a negative reaction in the insulin-acidity test are less categorical than are those of a positive response. After a bilateral vagotomy in man, a negative reaction is encountered frequently, but not invariably. Thus, in a stomach subjected to partial resection or to gastroenterostomy combined with vagotomy, a positive response of relatively small magnitude may be masked fortuitously by the intermittent partial neutralization of gastric acid by intestinal contents. Also, a negative reaction may be encountered in a patient within a few weeks after bilateral vagotomy, only to be followed some months later by an unequivocal positive reaction. Since the test does not differentiate a purely functional loss of vagal activity from a purely morphologic one, such a temporary postoperative negative reaction may result from trauma to the remaining untransected nerves. Subsequently, as this traumatic effect wears off, neural function will be recovered and a second insulin test will show a positive reaction. Finally, it occasionally happens that there is no rise in free acidity, following adequate hypoglycemia, but there is a significant increase in the total acidity curve, and therefore in the combined acidity. In conformity with the criteria set up for interpretation of this test, such a reaction must be reported as negative. However, future investigation may demonstrate this type of reaction to be a positive response of exceedingly low intensity, and this possibility must be remembered in the clinical utilization of the insulin-acidity test. In the absence of gastrovagal surgery, the incidence of negative responses is exceedingly rare. Although a few have been observed, no explanation for their occurrence has been advanced other than the possibility of their resulting from technical errors in performance or analysis of the test. In pouch dogs, a negative response indicates that the auxiliary stomach is entirely free from vagal influence either because of transection, as in the Heidenhain pouch, or because of degeneration in the isthmus of a Pavlov pouch (8, 11, 12).

REFERENCES

1. Babkin, B. P.: Triple mechanism of the chemical phase of gastric secretion, Am. J. Digest. Dis. 5: 467–472, 1938.
2. Ballem, C. M.; Noble, R. L., and Webster, D. R.: A new parasympathetic stimulant—ethyl 3:3 dimethylallyl barbituric acid, Canad. M.A.J. 58: 447–450, 1948.
3. Glass, G. B. J., and Boyd, L. J.: Three main components of human gastric mucin—dissolved mucoproteose, dissolved mucoprotein and mucoid of visible mucus: III. Preliminary data on physiological and clinical significance of separate quantitative determination of dissolved mucoproteose and dissolved mucoprotein in the gastric juice of man, Gastroenterology 12: 849–878, 1949.
4. Harrison, J. S., and McSwiney, B. A.: The chemical transmitter of motor impulses to the stomach, J. Physiol. 87: 79–86, 1936.
5. Hollander, F.: Studies in gastric secretion: II. A comparison of criteria of acidity used in this investigation, J. Biol. Chem. 91: 481–492, 1931.
6. Hollander, F.: The secretion of gastric mucus and hydrochloric acid in response to pilocarpine: Review of the literature, Gastroenterology 2: 201–211, 1944.
7. Hollander, F.: The insulin test for presence of intact nerve fibers after vagal operations for peptic ulcer, Gastroenterology 7: 607–614, 1946.
8. Hollander, F., and Jemerin, E. E.: Preparation of stomach pouch without interruption of vagal supply, Proc. Soc. Exper. Biol. & Med. 39: 87–90, 1938.
9. Hollander, F., and Stein, J.: Mucus, acid and water secretion in the stomach following injection of pilocarpine, Am. J. Physiol. 140: 136–147, 1943.
10. Ivy, A. C.; Grossman, M. I., and Bachrach, W. H.: *Peptic Ulcer* (Philadelphia: Blakiston Company, 1950).
11. Jemerin, E. E., and Hollander, F.: Gastric vagi in the dog: Erroneous assumption of uninterrupted vagal innervation in the Pavlov pouch, Proc. Soc. Exper. Biol. & Med. 38: 139–146, 1938.

12. Jemerin, E. E.; Hollander, F., and Weinstein, V. A.: Comparison of insulin and food as stimuli for differentiation of vagal and non-vagal stomach pouches, Gastroenterology 1: 500–512, 1943.
13. Malmejac, J., and Donnet, V.: Sur l'origine et le trajet des fibres chlorinergiques à destination gastrique contenues dans les nerfs splanchniques, Compt. rend. Soc. de biol. 133: 482, 1940.
14. Malmejac, J.; Donnet, V., and Monges, H.: Action des nerfs extrinsèques de l'estomac sur la motricité gastrique, Compt. rend. Soc. de biol. 133: 478–480, 1940.
15. Malmejac, J.; Donnet, V., and Monges, H.: Motricité gastrique et anastomoses intra-thoraciques entre les nerfs pneumogastriques chez le chien, Compt. rend. Soc. de biol. 133: 480–482, 1940.
16. Noble, R. L.: The stimulation and inhibition of gastric secretion in cats by barbiturate and thiourea derivatives, Canad. M.A.J. 60: 55–58, 1949.
17. Quigley, J. P., and Templeton, R. D.: Action of insulin on motility of the gastrointestinal tract: IV. Action on the stomach following double vagotomy, Am. J. Physiol. 91: 482–487, 1930.
18. Schachter, M.: Anesthesia and gastric secretion, Am. J. Physiol. 156: 248–255, 1949.
19. Sharick, P. R., and Campbell, D. A.: The gastric secretory responses to intravenously administered amino acid mixtures, Surgery 27: 396–402, 1950.
20. Stein, I. F., Jr., and Meyer, K. A.: Studies on vagotomy in treatment of peptic ulcer: III. Physiological aspect, Surg., Gynec. & Obst. 87: 188–196, 1948.
21. Thornton, T. F., Jr.; Storer, E. H., and Dragstedt, L. R.: Supradiaphragmatic section of the vagus nerves: Effect on gastric secretion and motility in patients with peptic ulcer, J.A.M.A. 130: 764–771, 1946.
22. Volborth, G. W., and Kudryavzeff, N. N.: The splanchnic nerve as a secretory nerve of the gastric glands, Am. J. Physiol. 81: 154–159, 1927.
23. Weinstein, V. A., and Hollander, F.: Is the insulin test always reliable? (editorial), Gastroenterology 14: 586–590, 1950.
24. Winkelstein, A.: Some physiological and pharmacological aspects of gastric secretory changes in peptic ulcer before and after partial gastrectomy, Tr. Am. Gastroenterol. A. 36: 206–214, 1933.
25. Zweig, M.; Meyer, K. A., and Steigmann, F.: The effect of intravenous protein hydrolysates on the stomach, Gastroenterology 12: 586–596, 1949.

Methods of Assaying Gastric Secretory Inhibitors*

M. I. GROSSMAN, *University of Illinois*

Reduction of gastric acidity is the keystone of present principles of peptic ulcer treatment, both medical and surgical. Because no drug other than atropine is sufficiently effective in reducing acid secretion to be clinically useful, reliance is placed on the much more inefficient process of neutralizing acid already secreted.

---

* This work was assisted in part by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service. Delbert Blickenstaff and Charles Rosiere assisted in this work.

Meanwhile the search for a useful inhibitor continues. As in the search for all specific therapeutic agents, so with the gastric secretory depressants, the availability of a rapid, reliable, quantitatively accurate, practical assay method is a potent factor in determining progress in the field.

Rats, cats and dogs have been utilized for assay of gastric secretory inhibitors. Both rat and cat assay methods are "acute" tests in which the animal is killed at the end of the test. In the rat method (6) the animal is fasted, anesthetized, the abdominal cavity is opened and a ligature is tied around the pyloric sphincter. Gastric secretion is continuous, requiring no stimulant. Half of the animals receive the "inhibitory" agent and the other half serve as controls. After several hours the stomachs are emptied, either by killing the animal and removing the stomach or by withdrawing the contents through a needle. In the latter instance periodic sampling may be done. The volume and acid concentration of the samples are measured and the values of the control group are compared with those of the "treats."

In the cat assay (14) the stomach is fistulized and gastric secretion stimulated by subcutaneous injection of a dose of histamine. After the response to this 1st dose of histamine has subsided, the inhibitory drug is given and the dose of histamine repeated. Control tests in which 2 doses of histamine are given but the inhibitor is omitted are performed for standardization of the method. Both the rat and the cat method have been shown to be capable of yielding reproducible quantitative results. However, it is generally recognized that assay methods using dogs are most reliable and efficient.

Comment by C. F. Code

Few satisfactory comparative data are available for support of this generalization. It is practically impossible today to express the results obtained by one method in terms of another.

The stomach of a dog may be chronically prepared for collection of gastric juice in various ways. A simple gastric fistula may be used (15–17), but this has the disadvantages (*a*) of allowing contamination of gastric juice with saliva or duodenal contents and (*b*) of the unpredictable changes that may take place due to presence of the vagal innervation. The latter objection also applies to the Pavlov type pouches. Heidenhain type pouches are free of these objections but present other problems: (*a*) The amount of juice secreted is likely to be low and therefore the accuracy of the measurement of volume of acid is correspondingly low. (*b*) To produce quantities of juice adequate for analysis, large doses of histamine must be used and this, as will be shown, reduces sensitivity of the assay. (*c*) The variability in secretory response of the Heidenhain pouch to a fixed dose of histamine is greater than that of the total gastric pouch (18).

Comment by C. F. Code

Ample juice for accurate measurement may be obtained from the large Heidenhain type of pouch in response to small doses of histamine. Mortality and morbidity are low in dogs with such pouches.

For these reasons we prefer and routinely use dogs with vagotomized pouches of the entire stomach. The preparation of dogs with total gastric pouches entails a high mortality rate in most hands. However, once the animal has survived the early postoperative period it usually has a normal life expectancy if a special diet is provided. This consists of commercial dog food (Pard), cooked ground horse meat (without bone), milk, ferrous sulfate and Pancreatin powder.

Types of test.—There are 2 general types of test, the "2-dose" (8) and the "continuous" test (7). In the 2-dose test, a dose of the stimulant, usually histamine, is given, and after the response to this has declined, a 2d dose of stimulant together with the inhibitor is given. In the continuous test, small doses of stimulant are given every 10 min throughout the test. When the secretory rate has reached a plateau, usually requiring 1–2 hr, the inhibitor is given and administration of stimulant and collection of juice is continued as long as desired. The only advantage of the 2-dose test is that it is easier to perform. It is decidedly less accurate than the continuous method and has the serious drawback of not giving information about the time of onset or rate of development and subsidence of the inhibition, important information which the continuous method does provide. With the 2-dose method it is possible to miss completely the inhibitory action of a drug if the relation of the time of injection of the inhibitor to the time of injection of the second dose of stimulator is not optimal. For almost all purposes the continuous type test is to be recommended.

Types of stimuli.—The following stimuli for gastric secretion can be used: histamine, food, insulin, parasympathomimetic drugs (methacholine, Urecholine, pilocarpine). Histamine works satisfactorily in any type of gastric fistula or pouch. Food stimuli (lean meat is best) work best in Heidenhain and Pavlov type pouches where the food acts on the pyloric part of the stomach and releases gastrin. In the total pouch dog, in which the food goes directly into the duodenum, the secretory response to feeding is much smaller. It is difficult to produce a sustained and level rate of secretion by use of a food stimulus, but by feeding small quantities at fixed short intervals, a relatively level rate can be achieved in Heidenhain pouch dogs. Insulin acts by producing hypoglycemia, which in turn acts as a central vagal stimulant, so that it is effective only in vagally innervated stomach preparations. It is not possible to produce sustained secretion with insulin. Parasympathomimetic drugs are strong stimulants of gastric secretion but produce undesirable side effects such as salivation, retching and defecation, and the secretory response tends to fall off after several hours (9).

Histamine is the preferred stimulant for general use because the response to it varies the least. There is no proof that histamine is a physiologic agent for stimulating gastric secretion, so the question arises whether it is a good drug to use in assaying inhibitors. The answer depends on what kind of inhibitor is being sought. If one wishes an inhibitor of a specific mechanism for stimulation of acid secretion, one should test against that specific stimulus. However, if one is searching for a general depressant of parietal cell activity (and this is the therapeutic ideal), the use of histamine is justified.

Comment by C. F. Code

Considerable evidence indicates that histamine plays a role in the normal secretory process.

When an inhibitor has been found and one wishes to work out the spectrum of its actions it is then necessary to test it against all of the types of stimuli which have been mentioned, using the various types of gastric fistulas and pouches listed. Ultimately the therapeutic value of an inhibitor can be determined only by tests in human subjects, using the information obtained from assays and toxicity studies in animals as a guide.

Types of inhibitors.—These fall into 2 broad groups: (1) synthetic drugs and plant alkaloids, and (2) extracts of animal origin including enterogastrone (7), urogastrone (8) and histaminase (11).

Routes of administration.—For routine screening of possible inhibitors, the intravenous route is probably best because it may detect substances which are inactive by other routes. E.g., with enterogastrone and urogastrone preparations about 10 times as large a dose is required intramuscularly as intravenously to produce comparable inhibition. Once an inhibitor has been found, one may wish to test it orally to determine whether it is active by this route. Occasionally one may wish to test an inhibitor which acts topically on the gastric mucosa.

CONTINUOUS HISTAMINE TEST IN TOTAL POUCH DOGS

The continuous histamine test in total pouch dogs (5, 7) has the greatest general usefulness.

Comment by C. F. Code

Continuous secretion in response to histamine is certainly very useful in dogs and I fully concur with the recommendation of it. The dogs, however, have not always had pouches of the entire stomach (5). Recently, too, the continuous secretion of gastric juice in rats following pyloric ligation has become a popular means of testing for inhibitor activity (6). Studies correlating different methods are needed.

a) *Individual test.*—The dogs, fasted for 12 hr or longer, are placed in stocks with neck restraints in which they have been trained to stand quietly. Perforated rubber catheters in plastic tripod holders (Fig. 1) are inserted in the stoma of the pouch, rubber funnels are fastened over the stoma by strings tied around the animal's body and a 50 cc Erlenmeyer flask is suspended with its mouth receiving the stem of the funnel. Histamine (see p. 178 for doses) is given subcutaneously in a fixed dose exactly every 10 min for the duration of the test, both before and after administration of the inhibitor. It has been shown (12) that this produces as high and as stable a secretory rate as when the same amount of histamine is given by continuous intravenous infusion (7). The entire contents of the Erlenmeyer flask are poured into a graduated cylinder every 20 min, the volume is recorded and an aliquot (5 cc), or the entire sample if it is small, is titrated with 0.03N NaOH, using p-dimethylaminoazobenzene and phenolphthalein as indicators. Electrometric titration or the indicators suggested by Hollander (13) may be used. The free acid output for the 20 min period, expressed in millimols, is calculated as

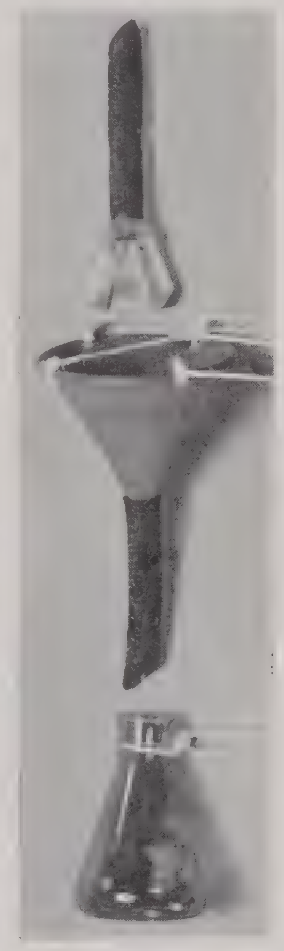

FIG. 1.—Collecting funnel, catheter, plastic tripod and Erlenmeyer flask used in collecting gastric juice from dogs with pouches of the entire stomach.

$$\frac{\text{buret reading (cc)} \times \text{normality of NaOH} \times \text{vol. of sample (cc)}}{\text{aliquot titrated (cc)}}$$

During the first 1–2 hr the secretory rate increases, then reaches a plateau which usually is maintained indefinitely (5, 7). Arbitrarily, 80 min may be taken as sufficient time for leveling off, and the three 20 min samples following this time are taken as the control level of secretion.

TABLE 1.—HOUR-TO-HOUR VARIABILITY OF SECRETORY RATE OVER 5 HR IN TOTAL POUCH DOGS GIVEN 0.0125 MG HISTAMINE EVERY 10 MIN

	COEFF. OF VAR., %		
DOG	Test 1	Test 2	Test 3
1	25	30	38
2	45	58	14
3	17	50	29
4	48	68	43
5	18	25	
6	23	33	
7	11	14	
8	14	45	
9	92	47	
10	36	78	

Mean for all tests—37.5%

TABLE 2.—DAY-TO-DAY VARIABILITY OF GASTRIC SECRETORY RESPONSE TO STANDARD DOSE OF HISTAMINE*

	SERIES I		SERIES II	
DOG	Mean ± Std. Error (mM/hr)	Coeff. of Var. (%)	Mean ± Std. Error (mM/hr)	Coeff. of Var. (%)
1	1.06 ± 0.17	52	1.39 ± 0.18	42
2	2.53 ± 0.36	45	3.91 ± 0.42	34
3	2.89 ± 0.64	70	1.95 ± 0.45	73
4	1.09 ± 0.11	32	2.61 ± 0.39	47
5	0.85 ± 0.15	57		
6	2.43 ± 0.37	49		
7	1.31 ± 0.18	44		
8	2.75 ± 0.34	39		
9	2.53 ± 0.15	19		
Mean		45.2		

* Histamine dihydrochloride subcutaneously every 10 min. Values are for the "control hour" as defined in the test, and each mean is derived from values for 10 tests over a 2 month period. Series I and II were separated by an interval of 2 months.

The constancy of the secretory rate from hour to hour may not be apparent if only a few tests are examined. E.g., in 29 control tests (no inhibitor was injected) on 6 total pouch dogs, using 0.0125 mg of histamine dihydrochloride every 10 min, we obtained the following mean values (with their standard errors) for the hourly output of free HCl in mM: 1.77 ± 0.26, 1.85 ± 0.23 and 1.84 ± 0.22. The secretory rate varied from 0.08 to 4.73 mM/hr and percentage deviation from the rate during the first hour was −91 to +257 with the least fluctuation in any test being −8%. The hour-to-hour variability in another series of control tests is given in Table 1. Table 2 shows data on the day-to-day variability in rate of secretion during the control hour. The variability of our results is greater than that reported by Code *et al.* (5) for Heidenhain pouch dogs, probably because we used considerably lower doses of histamine.

When the secretory rate has become stabilized, the inhibitor is injected and collections are continued as long as required in the individual case. It should be noted that some inhibitors do not show peak activity until several hours after injection (1), so, in the case of an unknown substance, observation should continue for at least 2 and preferably 3 hr after injection. The percentage inhibition for each hour after injection is calculated as

$$\frac{\text{free acid output for control hour (mM)} - \text{free acid output for postinjection hour (mM)}}{\text{free acid output for control hour (mM)}} \times 100$$

In most tests, average inhibition for the first 3 hr after injection is calculated to express the result of the test as a single figure.

b) Complete assay composed of several tests.—A single test on a single dog is insufficient to establish the activity of a drug. A number of tests at each of several dose levels must be done. In addition it is usually desirable to compare the unknown drug with a known drug which serves as a standard. To set up such a series of tests the principle of randomization is used so that on any 1 day each dog receives a different dose or a different drug. Assume that 4 dogs are being used for the assay. Then use 3 dosage levels for the unknown and 3 for the standard and use each dose of each drug on each dog. This would require 24 tests or 6 days (the dogs should not be used oftener than every other day); they could be randomized as follows:

Day / Dog	1	2	3	4	5	6
1	S_1	U_1	S_2	U_2	S_3	U_3
2	U_1	S_2	U_2	S_3	U_3	S_1
3	S_2	U_2	S_3	U_3	S_1	U_1
4	U_2	S_3	U_3	S_1	U_1	S_2

The data so obtained may be analyzed by calculating the mean inhibition for each dose level of each drug and fitting dose response lines graphically, using log-probability paper (Fig. 2) (1). If greater refinement of calculation is desired, the type of analysis of variance suggested by Bliss (4) for insulin assays may be used.

b) Histamine dosage.—Code *et al.* (5) showed that as the histamine dose is increased the variability in rate of secretion decreases, and at the same time the amount of inhibition produced by a fixed dose of inhibitor also decreases. Thus one must choose a level of histamine administration which will be high enough to give satisfactory stability of secretory rate and low enough to permit the inhibitors being studied to produce satisfactory inhibition. In total pouch dogs, histamine doses of 0.0125–0.0250 mg of dihydrochloride every 10 min are required to give adequate secretory rates.† Once a dose of histamine is selected it should be used for all dogs for all tests in a given assay series. Code *et al.* (5) suggested that the histamine dose be adjusted in each test so as to produce a secretory rate which is a certain percentage of the previously established maximal secretory rate for that dog. The basis for this suggestion was the observation by Gray *et al.* (7) that the degree of inhibition produced by a fixed dose of enterogastrone was inversely related to the control level of secretion. Gray did not present enough data to establish this point and Code's data merely showed that as the histamine dose (and therefore the control secretory rate) increased the percentage inhibition decreased. We (2) analyzed our data to determine whether fluctuations in inhibition could be correlated with fluctuations in control secretory rate when histamine dose and inhibitor dose were kept constant. We were

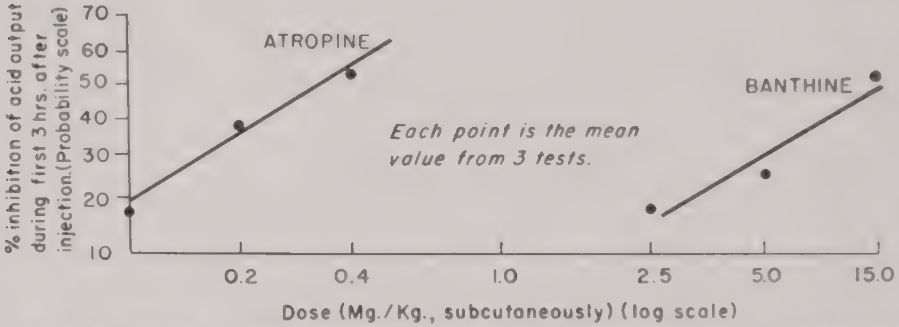

FIG. 2.—Dose-response lines for 2 drugs assayed in a randomized block scheme, using 3 dogs with total pouches and testing each dose of both drugs on each dog, totaling 18 tests run on 6 days.

---

† For conversion factors to equate the dihydrochloride and diphosphate salts of histamine, see Grossman, M. I.: Gastroenterology 15: 378, 1950.

unable to demonstrate such a correlation, but we did find a distinct inverse relationship between size of the histamine dose and the percentage inhibition (Fig. 3). Therefore we recommend that a fixed dose of histamine be used. However, the good linear dose-response relation which Code *et al.* have shown with their method of adjusting histamine-dose indicates that it, too, will give satisfactory results. Code *et al.* (5) also showed that above a certain secretory level, and therefore above a certain histamine dose level, percentage inhibition no longer tended to fall off with rising secretory rate.

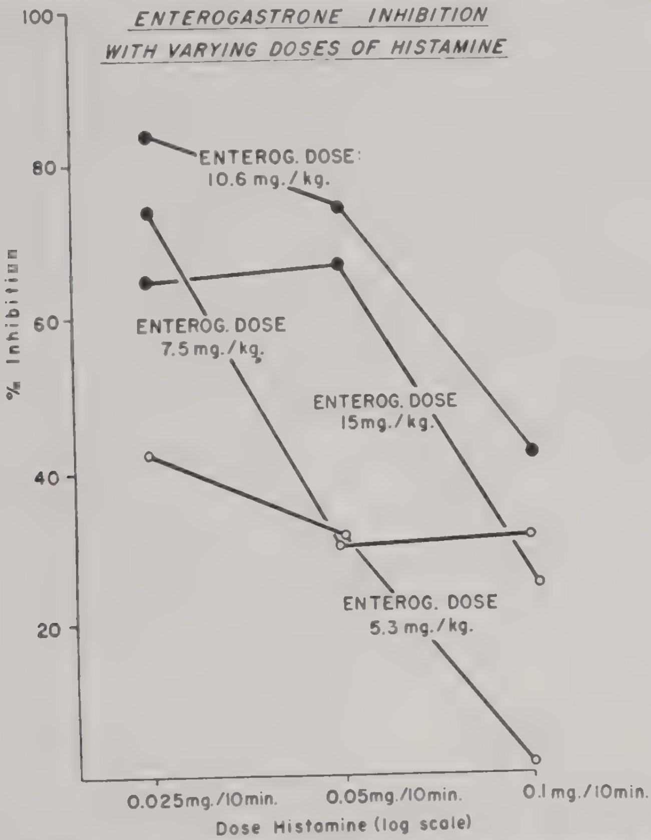

FIG. 3.—Relationship between dose of histamine used and percentage inhibition produced by various doses of an enterogastrone preparation. Each point is the mean value of tests on 3 dogs. There is a distinct inverse relation between histamine dosage and percentage inhibition.

Comment by C. F. Code

Full details of the analysis the author refers to here have not yet been published. Few data are given in reference (2). Small differences in control secretory rates would not produce significant differences in amounts of inhibition, but in my experience, doubling the output of a pouch from

25 to 50% of its maximal has constantly caused a significant reduction in the inhibition produced by some substances.

c) Pyrogens.—It is well known that pyrexia, however it is induced, inhibits secretion of acid by the stomach. It is important, therefore, in the study of gastric inhibitors to determine whether all or a part of the inhibition being observed is due to pyrogenic side effects. Synthetic drugs as well as biologic extracts should be studied from this point of view, although the latter are more likely to produce pyrexia. Measurement of the rectal temperature of the dogs each hour should be a routine part of every assay. We have studied purified bacterial pyrogen and Menkin's pyrexin to determine quantitatively the potency of pyrexia as a secretory inhibitor (3). The degree of pyrexia and percentage inhibition of secretion produced by graded doses of pyrogen is shown in Figure 4.

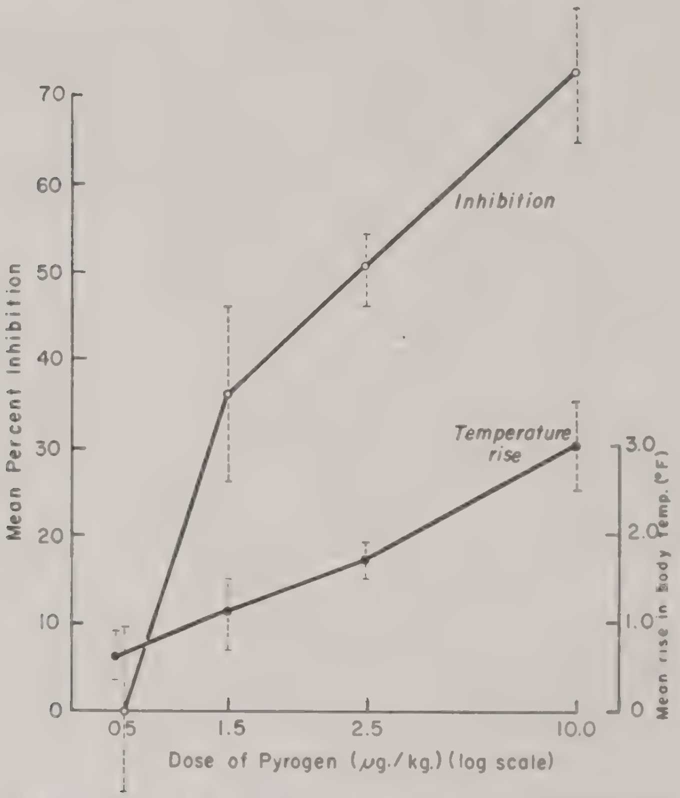

FIG. 4.—Mean rise in body temperature and mean percentage inhibition produced by graded doses of a bacterial pyrogen (Pyromen, Baxter). Each point is the mean of 4–7 tests; bars extending from the points represent standard errors of the means. Both percentage inhibition and temperature rise are calculated as mean value for the 3 hr following injection.

Only a small portion of the inhibition observed with an enterogastrone preparation could be attributed to pyrogenic activity (Fig. 5). Another helpful means of deciding whether the inhibitory action of a drug or extract is due solely to its pyrogen content is by studying the time course of the inhibition produced. On the average, inhibition does not occur during the first hour after injection of purified pyrogen (although pyrexia is usually maximal at this time), whereas the enterogastrone preparation produces significant inhibition during the first hour after injection (Fig. 6). In contrast, a preparation of chorionic gonadotrophin which we tested acted like pyrogen in that it did not produce inhibition until the second and later hours, and the degree of inhibition could be fully accounted for by the pyrexia it caused.

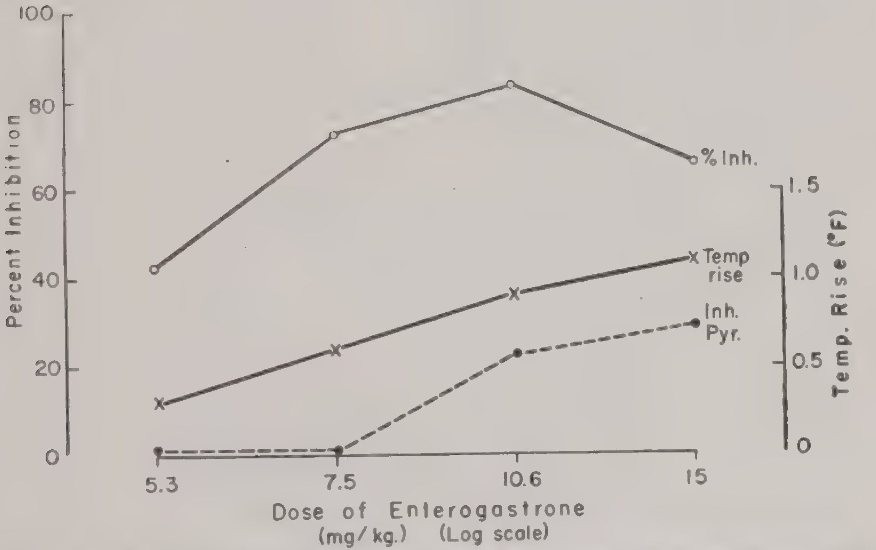

FIG. 5. Temperature rise and percentage inhibition of free acid secretion produced by graded doses of an enterogastrone preparation. Each value is the mean of 3 tests. Dotted line is the portion of observed inhibition of free acid secretion attributable to the effect of pyrogens, based on inhibition noted when purified pyrogens are used. (See Fig. 4.)

Other toxic actions besides pyrexia which may produce nonspecific inhibition of secretion are retching and vomiting (10), and profound hypotension also interferes with acid secretion by the stomach.

Another factor to be dealt with in the assay of biologic extracts is presence of stimulatory substances as well as inhibitors in the extracts. This phenomenon is illustrated in Figures 3 and 5, where it is shown that the highest dose of enterogastrone extract used produced less inhibition than the next smaller dose. This anomalous effect is frequently encountered with enterogastrone extracts and is attributed to the presence in them of stimulators. Some success has been achieved in removing these stimulators by chemical fractionation (6a).

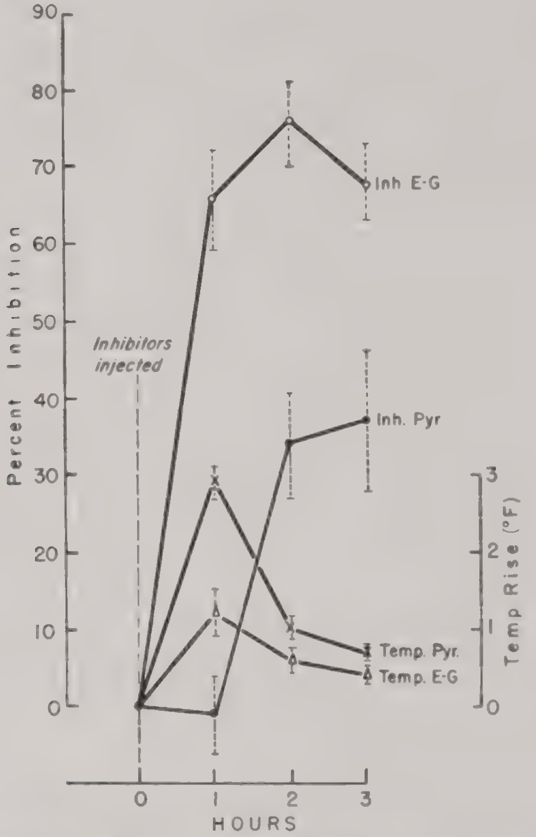

FIG. 6.—Comparison of time course of temperature rise and of secretory inhibition after injection of an enterogastrone preparation and a bacterial pyrogen. Note: (*a*) that enterogastrone produces less temperature rise but more secretory inhibition and (*b*) that with pyrogen there is a latent period of 1 hr between injection and beginning of secretory inhibition, whereas with enterogastrone secretory inhibition is near maximal in the 1st hour. Values for the tests with enterogastrone are the mean of 7 tests; those for pyrogen, 28 tests. Vertical lines extending from the points represent standard errors of the means.

REFERENCES

1. Benjamin, F. B.; Rosiere, C. E., and Grossman, M. I.: Comparison of the effectiveness of Banthine and atropine in depressing gastric acid secretion in man and the dog, Gastroenterology, 15: 727, 1950.
2. Blickenstaff, D., and Grossman, M. I.: Analysis of factors affecting the assay of gastric secretory depressants, Federation Proc. 8: 12, March 1949.

3. Blickenstaff, D., and Grossman, M. I.: A quantitative study of the reduction of gastric acid secretion associated with pyrexia, Am. J. Physiol. 160: 567, March, 1950.
4. Bliss, C. I., and Marks, H. P.: The biological assay of insulin: I. Some general considerations directed to increasing the precision of the curve relating dosage and graded response, Quart. J. Pharm. & Pharmacol. 12: 82, Jan.–Mar., 1939; II. The estimation of drug potency from a graded response, ibid. 12: 182, Apr.–June, 1939.
5. Code, C. F., *et al.:* A method for the quantitative determination of gastric secretory inhibition, Gastroenterology 13: 573, December, 1949.
6. Friedman, M. H. F., and Sandweiss, D. J.: A method for bio-assay for extracts which inhibit gastric secretion, Am. J. Digest. Dis. 13: 108, April, 1946.
6a. Gershbein, L. L.; Millen, H. M., and Ivy, A. C.: Fractionation of enterogastrone concentrates with n-butyl alcohol, Federation Proc. 8: 56, March, 1949.
7. Gray, J. S.; Bradley, W. B., and Ivy, A. C.: On the preparation and biological assay of enterogastrone, Am. J. Physiol. 118: 463, March, 1937.
8. Gray, J. S., *et al.:* Factors influencing the excretion of urogastrone, Am. J. Physiol. 134: 623, October, 1941.
9. Gray, J. S., and Ivy, A. C.: Effects of mecholyl on gastric secretion, Am. J. Physiol. 120: 705, December, 1937.
10. Grossman, M. I., *et al.:* The effect of nausea on gastric secretion and a study of the mechanism concerned, Gastroenterology 4: 347, April, 1945.
11. Grossman, M. I., and Robertson, C. R.: Inhibition by histaminase of gastric secretion in dogs, Am. J. Physiol. 153: 447, June, 1948.
12. Hanson, M. E.; Grossman, M. I., and Ivy, A. C.: Doses of histamine producing minimal and maximal gastric secretory responses in dog and man, Am. J. Physiol. 153: 242, May, 1948.
13. Hollander, F. H.: Studies in gastric secretion: II. A comparison of criteria of acidity used in this investigation, J. Biol. Chem. 91: 481, 1931.
14. Howat, H. T., and Schofield, B.: A double histamine test for inhibition of gastric secretion, J. Physiol. 107: 30P, March, 1948.
15. Kaulberz, J., *et al.:* Alterations in urogastrone excretion produced by extirpation of various endocrine glands, Am. J. Physiol. 150: 373, September, 1947.
16. Kaulberz, J., *et al.:* The effect of urine extract from thyroidectomized dogs on gastric secretion, Rev. Gastroenterol. 16: 257, March, 1949.
17. Kaulberz, J., *et al.:* The effect of urine extract from oophorectomized dogs on gastric secretion, Rev. Gastroenterol. 16: 254, March, 1949.
18. Lim, R. K. S.; Ivy, A. C., and McCarthy, J. E.: Contributions to the physiology of gastric secretion: I. Gastric secretion by local (mechanical and chemical) stimulation, Quart. J. Exper. Physiol. 15: 14, March, 1925.

Methods for in Vitro Study of Gastric Secretion

HORACE W. DAVENPORT, *University of Utah*

The fascinating problems posed by the secretion of hydrochloric acid by the gastric mucosa have been studied by many methods since Claude Bernard used his Prussian blue technique in an attempt to define the exact site of secretion. Pouch methods have been useful in elucidating the factors controlling secretion. However, the complexities of the blood supply to the stomach, the difficulties of obtaining pure acid secretion and the numerous cell types of the stomach severely limit the usefulness of Pavlov's methods in studying the mechanism by which the secretion is formed. Although some important information has been obtained from pouch preparations (6, 22, 25, 26, 28), workers have been turning to isolated preparations of the stomach in which the metabolism of the gastric mucosa can be studied together with its ability to secrete and in which the experimental conditions can be modified to a greater degree and in a more predictable manner than in the whole animal.

Comment by L. E. Edwards

The early pouch methods have been very useful as a guide in gastric studies. In vitro methods have an important role in further studies of gastric secretion, particularly in relation to the intermediate processes involved in secretion. In vitro gastric studies play the same part in gastric physiology as tissue metabolism techniques (Warburg) play in the physiology of metabolism. Neither type of study alone can give complete information.

PREPARATIONS WITH INTACT BLOOD SUPPLY

To study the metabolism of the secreting mucosa, several groups of workers have performed acute experiments in anesthetized dogs in which the stomach is delivered through an abdominal wound. The stomach is opened, and the arterial supply and venous drainage of a selected portion of the mucosa are isolated. The secretion is collected and, if required, samples of arterial and venous blood are obtained. Mann *et al.* (31) used this method for study of chloride exchange from the blood to the secretion. Rehm *et al.* (33-36) isolated the mucosa and muscularis in a clamp formed of Lucite rings and bathed each side of the tissue in saline solutions. Several types of electrodes designed for measurement of gastric potentials and current production were placed in the saline solutions, permitting study of the electrical potentials accompanying secretion. A few observations have also been made by these workers on the gas and carbohydrate metabolism of the secreting mucosa. In these experiments venous blood samples have been taken and the mucosa analyzed at the end of the experimental period.

The numerous disadvantages of this type of preparation limit its usefulness. For most metabolic studies it is necessary to know the rate of blood flow through the stomach so that total metabolic exchange can be calculated. The blood flow can be measured directly by the elaborate apparatus used by Still *et al.* (38) in their study of the pancreas, but such methods have apparently not been applied to the stomach. Calculations of the blood flow through the stomach based on the Fick principle are extremely inaccurate. The additional obvious disadvantages of the method preclude its use in obtaining the vast amount of information necessary before the mechanism of secretion can be understood, but the method may give results of an indicative nature.

Comment by L. E. Edwards

Rehm's method most nearly bridges the gap between in vitro and whole animal studies of gastric metabolism. It has been put to excellent use in measuring potential changes.

Houssay (29) has developed a preparation in which the whole excised stomach of the dog is perfused with blood, but the details are not available.

PREPARATIONS IN WHICH MUCOSA SEPARATES 2 CHAMBERS

The earliest method of studying the gastric mucosa in vitro was described by Delrue in 1930 (12–15), and modifications have been used by many others (1, 2, 10, 11, 16–20, 23, 24, 32). Figure 1 illustrates one form of the apparatus. The stomach is removed from the animal, usually an amphibian, and the mucosa separated from the muscularis. A piece of the mucosa is fastened in a form of clamp which holds it firmly but exposes both sides of the tissue. The clamp is then fastened between 2 glass or plastic chambers. The chamber on the muscularis side is filled with nutrient solution, plasma or buffered physiologic saline solution, and O_2 or an O_2-CO_2 mixture is bubbled through it. The chamber on the secreting side is filled with buffered or unbuffered saline solution. The acid production is measured by following the pH of the second solution or by removing samples for titration.

Comment by L. E. Edwards

In vitro methods are essential for the study of many factors, e.g., potential differences, O_2 consumption of secretory tissue, secretion pressure, intermediate metabolites and permeability. None of the following methods is suitable for measuring all factors simultaneously, but each is adapted to some specific problem.

This type of apparatus has been used for many purposes, and appropriate modifications have been made in its structure. It is

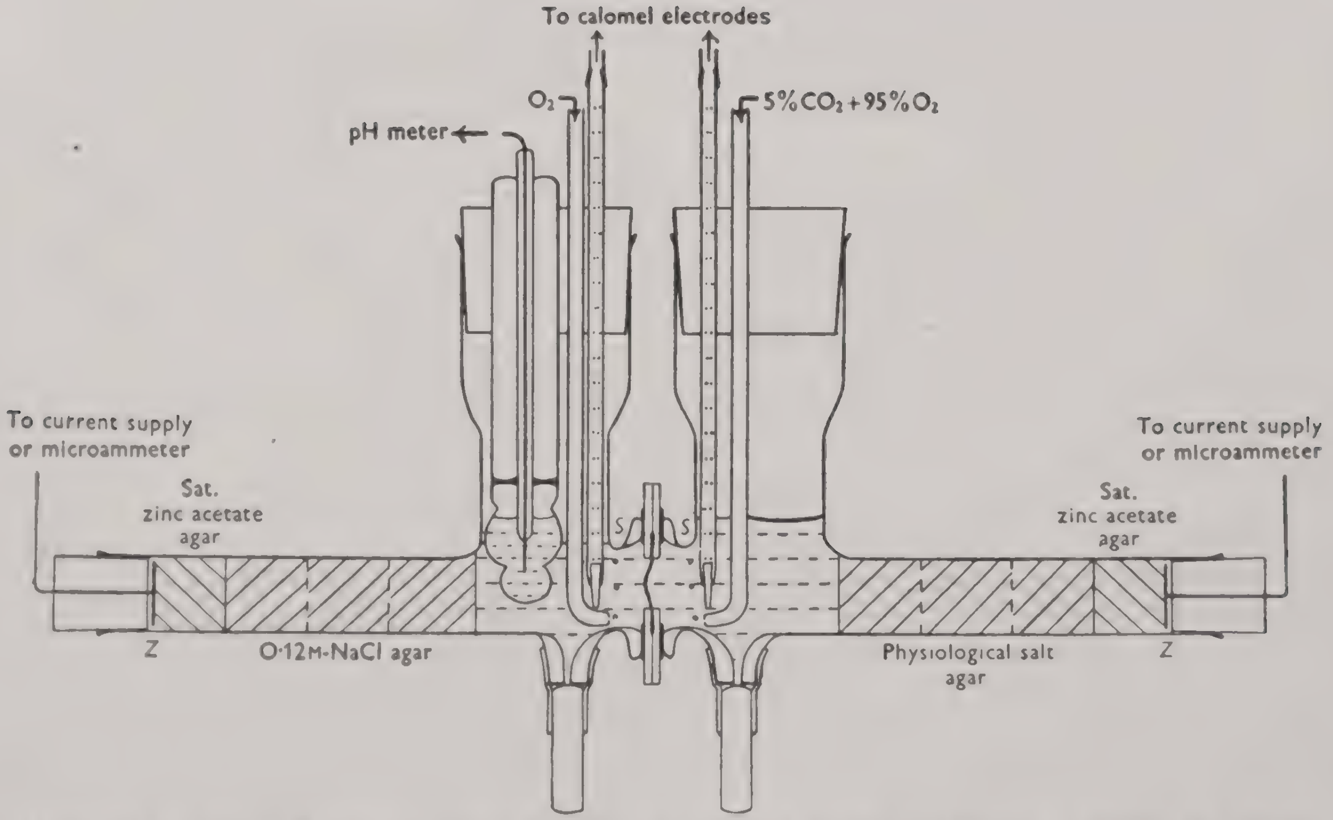
To calomel electrodes
O_2
5% CO_2 + 95% O_2
pH meter
To current supply or microammeter
Sat. zinc acetate agar
S
S
Z
0·12M-NaCl agar
Physiological salt agar
Z
To current supply or microammeter
Sat. zinc acetate agar

FIG. 1.—Apparatus for study of relation between secretion and electrical energy production in isolated frog mucosa. The mucosa, held by a clamp, separates 2 chambers containing physiologic saline solution. Gas mixtures bubble through the solutions and the potential is measured by the 2 vertical agar bridges. Additional oblique bridges attach at *SS*. The glass electrode in the left-hand chamber measures the pH of the secretion. The 2 horizontal electrodes are used for measuring energy output or for establishing potential gradients. (From Crane *et al.* (1), by permission.)

particularly useful for study of the electrical events associated with secretion. By nonpolarizable electrodes placed in the 2 chambers the potential developed by the mucosa can be measured (1, 2). The large electrodes shown in Figure 1 have been used by Davies *et al.* for study of the power output of the mucosa under impressed potential gradients. They have also attached a manometer to the chambers to measure the mucosa's ability to secrete against hydrostatic pressure. The method can also be used for the study of the effects of changes in composition of the nutrient solution (23, 24) and the effects of various substrates and inhibitors.

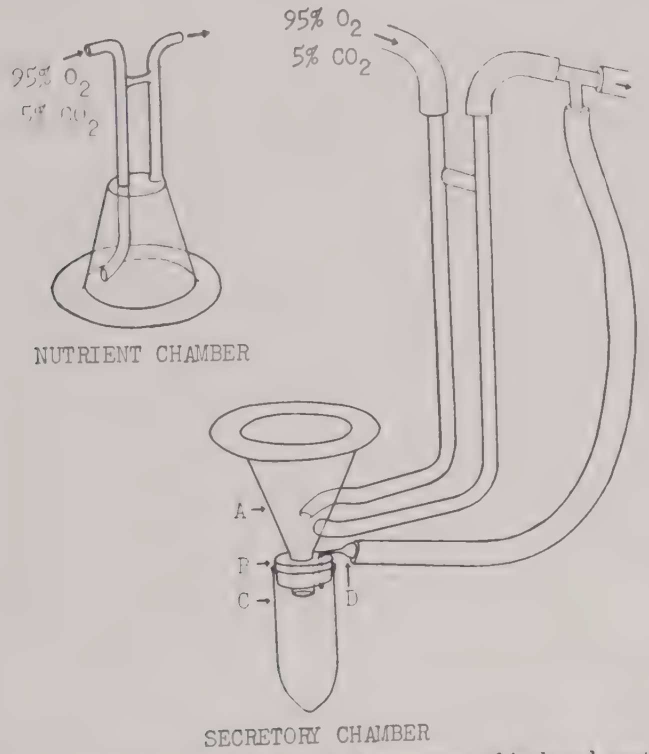

FIG. 2.—Double chambers for study of secretion of isolated gastric mucosa. Mucosa is placed between the chambers, secretory side down, and the chambers are held together by a spring clamp. Nutrient solution is placed in the upper vessel, and gas mixtures are blown through both vessels. The secretion collects in the tube at the bottom. (From Edwards and Edwards (20).)

In a different type of chamber, used by Edwards and Edwards (16–20) (Fig. 2), the mucosa is clamped between 2 conical vessels with the secreting side down. Nutrient solution is placed over the mucosa in the upper vessel, and the secretion drains into the collecting tube attached to the lower vessel. Gas mixtures are blown through the vessels, and the whole apparatus is shaken in a water bath.

With these methods it is impossible to measure the respiration of the mucosa, and it is difficult to measure other metabolic changes accompanying secretion. No worker using the methods has presented proof that the mucosa is performing under optimal conditions, and when the low rates of secretion obtained in many instances are considered it appears likely that the method has several inherent serious objections.

Comment by L. E. Edwards

Both whole blood and serum have been used as the nutrient medium. These decrease, if they do not entirely eliminate, the pronounced permeability changes found with synthetic media.

SACS OF EXCISED STOMACH

Davies (10) has made many observations on the acid secretion produced by sacs of excised frog stomachs and has occasionally used toad, mouse and sheep stomachs. In all preparations the mucosa is stripped from the submucosa. For experiments with open tissue the mucosa is cut along the greater or lesser curvature and placed directly in a Warburg flask. Tied tissue is used when collection of the secretion is required. The pyloric end of the stomach is tied with silk thread and the lumen emptied by gentle stroking. The cardiac end is tied, and the stomach is placed in a bicarbonate-buffered saline solution contained in the cup of a Warburg vessel.

Davies (10) has also used a preparation of amphibian mucosa in which the stomach is everted so that the secretion collects on the outside of the tissue. When the stomach is turned inside out the lumen is not large enough to contain sufficient nutrient solution. To supply nutrient solution a tiny glass U-tube is made, and a 4 ml balloon of very thin rubber is tied over 1 arm. A clean intact mucosa is turned inside out, and after the pyloric end is tied the cardiac end is attached to the free arm of the U-tube. With a syringe the nutrient solution is injected into the balloon and the air in the balloon sucked out. The puncture hole in the balloon is tied, and the preparation is placed in a Warburg cup. When the cup is shaken the fluid in the balloon moves back and forth through the U-tube, and in this way the muscularis side of the mucosa is brought into contact with somewhat more than 4 ml of fluid. Although the preparation is ingenious, it has no advantages over that described in the previous paragraph.

All of these preparations are incubated with shaking in a water bath at the desired temperature. The fluid is equilibrated with a gas mixture at atmospheric pressure. At the end of the incubation period the various fluids are collected, weighed and analyzed by standard manometric or chemical methods.

Davenport *et al.* (5, 7–9) have used preparations of excised mouse stomachs.

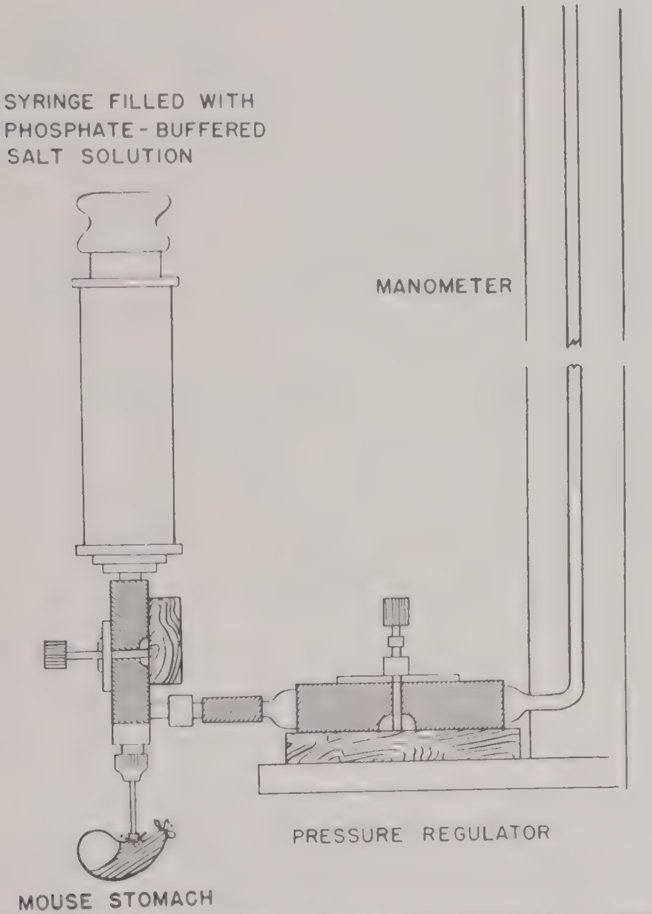
SYRINGE FILLED WITH
PHOSPHATE - BUFFERED
SALT SOLUTION
MANOMETER
PRESSURE REGULATOR
MOUSE STOMACH

Fig. 3.—Apparatus used for washing out and inflating a mouse stomach.

PROCEDURE

A mouse is decapitated, and the esophagus is divided high in the thorax and pulled through the diaphragm. The stomach is freed and the duodenum divided. The esophagus is slipped over a blunt hypodermic needle (Fig. 3), and the stomach is washed out with phosphate-buffered saline solution contained in the syringe. The pylorus is tied, and the stomach inflated with saline solution until pressure in the stomach is 75 cm H_2O. The esophagus is tied, the stomach placed in 4 ml of the saline solution in a weighing bottle and the bottle placed in a brass bomb (Fig. 4). Oxygen is blown through the bomb and the O_2 partial pressure raised to 3200 mm Hg. The bomb is incubated with shaking in a water bath at 38 C for 1 hr. The time elapsing between decapitation and placing of the bomb in the water bath is about 2 min.

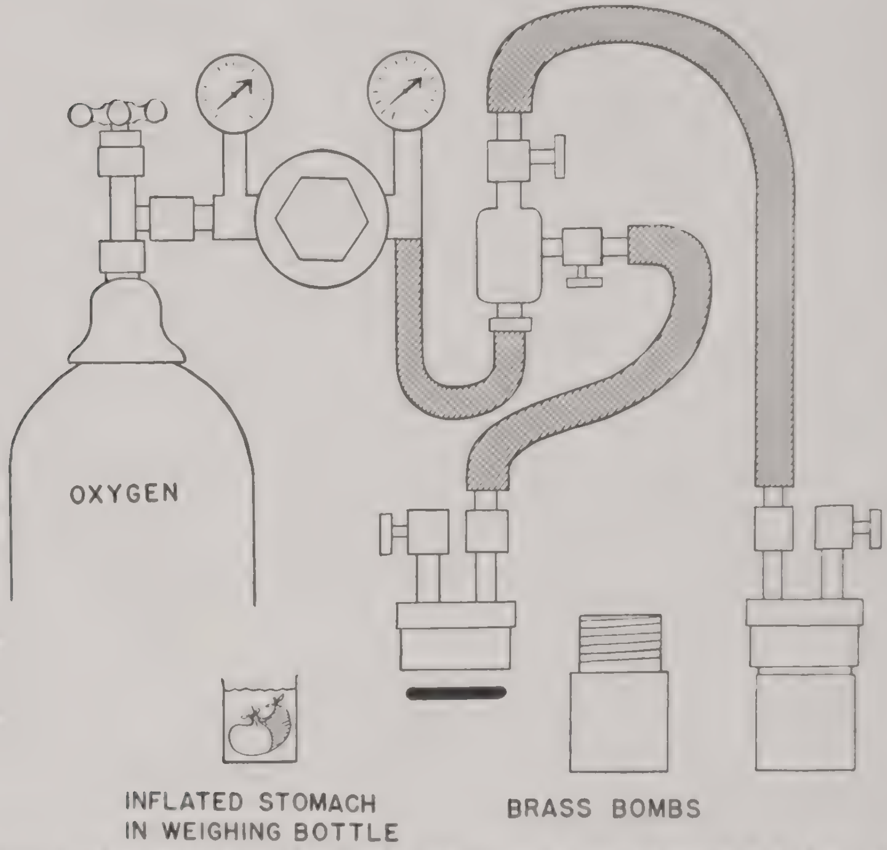

FIG. 4.—Apparatus used for incubating inflated mouse stomachs at high O_2 pressures. The bombs are shaken in a water bath at 38 C.

Comment by L. E. Edwards

The high O_2 pressure required means working very near the toxic O_2 level. The permeability changes of the tissue should be checked in this work.

At the end of the period of incubation the contents of the stomach are placed in a 5 ml crucible and weighed. The fluid is placed in contact with a small glass electrode and a calomel half-cell which are connected with a pH meter. A fine tube blows CO_2-free air through the solution for stirring, and the tip of an ultramicroburet dips beneath the fluid surface. The pH of the fluid is read and titrated with standard NaOH to the initial pH of the saline solution. The fluid is then analyzed for lactic acid. It has been proved that the total acid found minus the lactic acid is inorganic acid secreted. The secreting part of the stomach is dried and weighed or used for chemical analysis or cytologic study.

Extensive studies on the conditions affecting the rate of secretion by these preparations have been completed (5). The saline solution in which the stomach is bathed has the following composition: NaCl, 118 mM/l; KCl, 4.8 mM/l; $CaCl_2$, 1.3 mM/l; $MgSO_4$, 1.2 mM/l; KH_2PO_4, 1.2 mM/l, and Na_2HPO_4 brought to pH 7.4 with HCl, 16 mM/l. Small changes from these concentrations are tolerated, but gross changes reduce the rate of secretion. Because the tissue is thick, the rate of secretion is exceedingly sensitive to the partial pressure of O_2, and the optimal pressure is about 3200 mm Hg. Glucose is the best substrate, and its optimal concentration is between 15 and 30 mM/l. Pyruvate and lactate can partially replace glucose as a substrate, but no other effective substrate has been found. The rate of secretion within limits rises with increasing distention of the stomach. The optimal time of incubation is 1 hr, for the fall in pH below 2.5 which occurs at longer incubation periods stops the accumulation of acid. Secretion is stimulated by carbaminoylcholine at an optimal concentration of 0.1 mg %, and it is also stimulated by histamine. Histologic observations show that the cells of the stomach are well preserved during incubation.

Advantages of the sac methods over the 2 chamber methods are that the metabolic processes of the mucosa and the rate of secretion can be studied simultaneously. In addition, numerous reproducible observations can be made on the same stomach of the effects of hormones, substrates, inhibitors, etc. Because in Davenport's method the tissue is incubated under high gas pressure an inconvenient but not insuperable complication is introduced. Gas exchanges cannot be measured by the Barcroft-Warburg techniques; instead a high pressure apparatus such as that designed by Stadie and Riggs (37) must be used.

The methods described here can also be used for the study of pepsin and mucin secretion, but few investigations of this type have been made.

ENZYME LOCALIZATION

The stomach contains many different types of cells, and the results of metabolic studies made on the whole mucosa describe the sum of the metabolism of all cell types. Consequently, methods are needed by means of which specific parts of the total metabolism can be assigned to specific cells. Entirely satisfactory methods have not been found for accomplishing this, and some published conclusions rest on assumptions of doubtful validity. E.g., Davies (10) found that the rates of acid secretion and O_2 consumption in isolated frog stomachs increase when histamine is added to the preparation. On the assumptions that the extra O_2 consumption is used entirely for elaborating extra acid secreted and that none of the resting O_2 consumption is used more efficiently in the presence of histamine, Davies calculated the ratio of O_2 consumed to acid secreted. Several important theoretical conclusions are based on this calculation, but the conclusions would be more certain if it were possible to demonstrate that the ratio holds under many diverse circumstances.

Comment by L. E. Edwards

This has been the usual method of determining changes in metabolism caused by a given drug or condition. Although open to question, it does give an indication and a starting point for further experimentation.

Another method of attempting to separate the various parts of the stomach's metabolism is histologic localization of enzymes, substrates and electrolytes. The freezing-drying method, in which the mucosa is frozen in situ and then dried in vacuo at very low temperatures, has been used (27). However, the conclusion based on these studies, that acid is secreted as a protein complex which is hydrolyzed in the lumens of the glands, is almost certainly incorrect, and this makes its usefulness questionable.

Comment by L. E. Edwards

The evidence supporting this statement, i.e., that the conclusion is "almost certainly incorrect," is not given.

The older literature on the secretory processes of the stomach contains many papers dealing with the use of dyes, but for technical reasons the results are almost uniformly indecisive. More modern work has been based on attempts to establish correlation between the concentration of a substance which can be accurately measured and the frequency of occurrence of a particular type of cell. If it can be proved that an enzyme or other substance is present in a certain type of cell, the presumption that the substance is a part of the metabolic mechanism of the cell is a reasonable one.

Linderstrom-Lang and his colleagues (21, 30) have been most successful in demonstrating the cellular locus of enzymes in the stomach. In their methods a cylinder of mucosa whose axis is normal to the surface of the mucosa is cut with a cork borer. A smaller cork borer is used to cut a 2d cylinder from within the 1st. The smaller cylinder is then cut by a freezing microtome into sections of uniform thickness parallel to the surface of the mucosa. These sections are analyzed, and the results of the analysis are plotted as a function of the distance from the surface of the mucosa. The annulus of mucosa remaining after the smaller cylinder is cut from the larger one is fixed and embedded. Serial sections are cut in planes parallel to the surface of the mucosa. These sections are stained and their cell populations estimated by counting a sufficient number of microscopic fields. Finally, the estimated number of cells of various types are plotted as a function of the distance from the surface of the mucosa, and the curves obtained by cell counting and by analysis are compared. A correlation between the curves is taken as evidence that the substance is contained in a particular type of cell.

There are formidable technical difficulties in this method. Those involved in analysis of the tiny sections of tissue have been solved by the brilliant series of ultramicromethods for which Linderstrom-Lang is famous. Those involved in the histologic work are more serious, since corrections must be made for changes in the dimensions of the tissue occurring during fixing and embedding. Linderstrom-Lang has apparently also solved this problem, but few persons who have not worked with him have dared to use the method.

A less versatile method which avoids the histologic difficulties has been used to demonstrate the presence of carbonic anhydrase in parietal cells and cells of the surface epithelium (3, 4). In this work, cylinders of mucosa were cut on a freezing microtome in planes parallel to the surface of the mucosa. Cutting was continued until 3 consecutive uniform sections were obtained. The first and last section were analyzed for carbonic anhydrase, and the middle section was placed on a glass slide, fixed and stained. The cells of the fixed section were counted, and the enzyme concentration of the first and last sections was plotted against the cell populations. Good correlations were found between the enzyme concentrations and the frequency of surface and parietal cells.

It appears likely that much of the future work on the secretory processes of the stomach will consist of attempts to discover and analyze specific reactions occurring in the mucosa. In the course of this type of investigation it may be possible to construct hypotheses concerning the role of the reactions in the secretory processes. Before these hypotheses can be accepted it must be demonstrated that the reactions being studied actually occur in the cells known to form the secretion. Consequently, it is probable that the methods of enzyme localization will be frequently used.

Comment by L. E. Edwards

Work on enzymes is greatly hampered by the number of cell types involved. More work should be done to locate or isolate the various cell types.

REFERENCES

1. Crane, E. E.; Davies, R. E., and Longmuir, N. M.: Relations between hydrochloric acid secretion and electrical phenomena in frog gastric mucosa, Biochem. J. 43: 321, 1948.
2. Crane, E. E.; Davies, R. E., and Longmuir, N. M.: The effect of electric current on HCl secretion by isolated frog gastric mucosa, Biochem. J. 43: 336, 1948.
3. Davenport, H. W.: Gastric carbonic anhydrase, J. Physiol. 97: 32, 1939.
4. Davenport, H. W.: Gastric carbonic anhydrase in dogs, Am. J. Physiol. 128: 725, 1940.
5. Davenport, H. W., and Chavre, V. J.: Conditions affecting acid secretion by mouse stomachs in vitro, Gastroenterology, 15: 467, 1950.
6. Davenport, H. W., and Fisher, R. B.: The mechanism of the secretion of acid by the gastric mucosa, Am. J. Physiol. 131: 165, 1940.
7. Davenport, H. W., and Jensen, V.: The secretion of acid by the mouse stomach in vitro, Gastroenterology 11: 227, 1948.
8. Davenport, H. W., and Jensen, V.: Observations on the secretion of acid by the mouse stomach in vitro, Gastroenterology 12: 630, 1949.
9. Davenport, H. W., and Jones, B.: Thiamine deficiency, pyruvate metabolism and acid secretion in mouse stomachs in vitro, Gastroenterology 13: 235, 1949.
10. Davies, R. E.: Hydrochloric acid production by isolated gastric mucosa, Biochem. J. 42: 609, 1948.
11. Davies, R. E., and Longmuir, N. M.: Production of ulcers in isolated frog gastric mucosa, Biochem. J. 42: 621, 1948.
12. Delrue, G.: Study of the acid secretion of the stomach; secretion of the isolated mucosa of the frog stomach, Arch. internat. de physiol. 33: 196, 1930.
13. Delrue, G.: Acid secretion by the isolated mucosa of the frog stomach, Compt. rend. Soc. de biol. 105: 42, 1930.
14. Delrue, G.: Acid secretion by the isolated mucosa of the frog stomach, Arch. internat. de physiol. 36: 129, 1933.
15. Delrue, G., and Lacquet, A. B.: Study of the acid secretion of the stomach; effect of alkalosis on the acid secretion in the dog, Arch. internat. de physiol. 39: 295, 1934.
16. Edwards, L. E., and Edwards, C. T.: A method for the study of gastric secretion in vitro, Federation Proc. 7: 30, 1948.
17. Edwards, C. T., and Edwards, L. E.: Factors influencing in vitro secretion of pepsin, Federation Proc. 7: 30, 1948.
18. Edwards, C. T., and Edwards, L. E.: Effect of certain physical factors on the in vitro gastric secretion of the dog, Federation Proc. 8: 39, 1949
19. Edwards, L. E., and Edwards, C. T.: Acid secretion by gastric mucosa of the bull frog in vitro, Federation Proc. 8: 40, 1949.
20. Edwards, L. E., and Edwards, C. T.: Personal communication
21. Glick, D.: Urease in the human stomach with respect to acid secretion in ulcer and cancer, J. Nat. Cancer Inst. 10: 321, 1949.

22. Gray, J. S.: The physiology of the parietal cell with special reference to the formation of acid, Gastroenterology 1: 390, 1943.
23. Gray, J. S., and Adkison, J. L.: The effect of inorganic ions on gastric secretion in vitro, Am. J. Physiol. 134: 27, 1941.
24. Gray, J. S.; Adkison, J. L., and Zelle, K.: The in vitro secretion of acid by the gastric mucosa of the frog, Am. J. Physiol. 130: 327, 1940.
25. Gray, J. S., and Bucher, G. R.: The composition of gastric juice as a function of the rate of secretion, Am. J. Physiol. 133: 542, 1941.
26. Gray, J. S.; Bucher, G. R., and Harman, H. H.: The relationships between total, acid and neutral chlorides of gastric juice, Am. J. Physiol. 132: 504, 1941.
27. Hoerr, N. L., and Bensley, R. R.: Cytological studies by the Altmann-Gersh freezing-drying method: The mechanism of hydrochloric acid secretion in the gastric mucosa, Anat. Rec. 65: 417, 1936.
28. Hollander, F.: The chemistry and mechanics of hydrochloric acid formation in the stomach, Gastroenterology 1: 401, 1943.
29. Houssay, B.: Personal communication.
30. Linderstrom-Lang, K.: Distribution of enzymes in tissues and cells, Harvey Lect. 34: 214, 1939.
31. Mann, F. D.; Grindlay, J. H., and Mann, F. C.: The withdrawal of chloride from the blood by the gastric glands, Am. J. Digest. Dis. 8: 415, 1941.
32. Patterson, W. B., and Stettin, D.: A study of gastric HCl formation, Science 109: 256, 1949.
33. Rehm, W. S.: Electrical energy output of the resting stomach as determined by shunting its potential, Am. J. Physiol. 139: 1, 1943.
34. Rehm, W. S.: Positive injury potentials of the stomach, Am. J. Physiol. 140: 720, 1944.
35. Rehm, W. S.: The effect of electric current on gastric secretion and potential, Am. J. Physiol. 144: 115, 1945.
36. Rehm, W. S.: Evidence that the major portion of the gastric potential originates between the submucosa and mucosa, Am. J. Physiol. 147: 69, 1946.
37. Stadie, W. C., and Riggs, B. C.: An apparatus for the determination of the gaseous metabolism of surviving tissues in vitro at high pressures of oxygen, J. Biol. Chem. 154: 669, 1944.
38. Still, E. U.; Bennett, A. L., and Scott, V. B.: A study of the metabolic activity of the pancreas, Am. J. Physiol. 106: 509, 1933.

SECTION IV

Tissue Culture Methods

Associate Editor, *C. M. Pomerat*

INTRODUCTION

In 1927, R. G. Harrison summarized the scientific advances which had been made with the use of tissue culture methods during the 2 decades since their introduction in the field of experimental biology. These positive accomplishments consisted of:

1. A direct ocular demonstration of the embryological basis of the neurone concept.
2. A crucial proof of the myogenic theory of the heart beat.
3. A demonstration of the potential immortality and indefinite power of reproduction of somatic cells.
4. The discovery that dissociated somatic cells of animals having at least the degree of organization found in sponges and coelenterates can reconstitute themselves into normal viable individuals by a process resembling ontogeny.
5. A means for the exact study of growth in pure lines of cells, conditions influencing the process and the chemical nature of the medium required.
6. The discovery of numerous facts with regard to cell transformations that go a long way toward the solution of the vexed question of the genetic relations of blood and connective tissue elements in favor of the monogenetic or unitarian theory.
7. Important evidence regarding cell movement, the behavior of mitochondria, the mode of connection between tissue elements and their differentiation and dedifferentiation.
8. The opening of the process of cell division to direct observation, through which the peculiar movements of the cytoplasm, the duration of nuclear phases, the periodicity of the process and the influence of various external conditions upon it have been studied.
9. The discovery that tumor cells have the power of indefinite multiplication outside the body without losing their properties and that normal

somatic cells may, through proper treatment, be rendered capable of producing malignant tumors when reintroduced into the body of an animal.

10. The direct observation of the behavior of tissue cells toward micro-organisms and the conditions under which certain immune reactions occur.

11. The opening of tissue cells to the direct action of drugs whereby the influence of the latter on the living protoplasm may be studied.

The closing years of the second 20 year period in the history of tissue culture technique had seen some reduction in its use, but the study of cells cultivated in vitro was greatly stimulated as a result of a Conference on Tissue Culture held at Hershey, Pa., in 1946. This meeting, sponsored by the Committee on Growth of the National Research Council Acting for the American Cancer Society, led to the formation of the Tissue Culture Association. In 1948, European workers founded a Tissue Culture Club. Among the major objectives of the American organization have been: (1) the sponsorship of commercial production of culture media, (2) the preparation of a cross-index of the bibliography of tissue culture which will include approximately 15,000 titles, (3) the establishment of a training course for students at the doctoral level, and (4) the organization of meetings for the exchange of information.

Tissue culture technique has continued to increase fundamental knowledge regarding the genetic relation of normal and malignant cells, cell movements, the behavior of organells and the reaction of cells to micro-organisms and drugs. Of special interest during recent years have been: (1) Renewed efforts to determine the composition of synthetic media for plant and animal cells. (2) The simplification of methods for preparing biologic media by means of lyophilization, storage at low temperatures, the use of antibiotics and improvement of methods of filtration. (3) Development of clones of pure strain from isolated single cells. (4) The technique for cell suspension, replicate and mass culture suitable for quantitative biochemical study. (5) Cultivation of small but entire organs such as bones and endocrine glands. (6) Improvement of culture methods adapted for studies of cell physiology and drug assay. (7) Development of procedures for studying the effect of biologic radiations on cells. (8) The use of phase contrast optics for observations on living cells especially in combination with time-lapse cinematography. (9) The study of fine structure of cells grown in vitro with electron microscopy. These data are being correlated with information obtained on living cells with phase microscopy. (10) Increased effort in using tissue culture methods for diagnostic and experimental pathology. (11) Observations on intracellular parasitic relations with special reference to viruses, rickettsias and protozoa.

Renewed intense activity in tissue culture work is in the process of providing important biologic data, but current effort is particularly characterized by the development of many highly promising technical advances in the design of apparatus and procedures. This may, in part, account for the fact that in 1950, new editions were produced of the excellent books dealing with tissue culture technique both by Parker and by Cameron. As a result of the publication of these important sources of information, this section on tissue culture methods is brief and limited to items of particular interest to workers in the medical field or procedures requiring more extensive treatment than is accorded them in the collected literature.

—C. M. Pomerat

TISSUE CULTURE MEDIA

I. APPROACHES TO DEVELOPMENT OF SYNTHETIC MEDIA FOR THE ANIMAL CELL

Introduction

PHILIP R. WHITE, *Institute for Cancer Research, Philadelphia*

Nutrients for tissue cultures are of 2 types. They are either (1) made up largely of materials of biologic origin, more or less duplicable by adherence to strict empiric rules in their preparation, but not subject to exact chemical definition because of their derivation from complex sources, or (2) made up of chemically defined, preferably synthetic ingredients which make them precisely duplicable and subject to easy and exact manipulation. The former represent the classic nutrients developed by Harrison, Burrows, Carrel, Baker, Drew, Doljanski, Simms, Fischer and others. They have given, and continue to give, important results in many fields of investigation. They do not, however, permit unequivocal answers to questions of nutrition, metabolism, immunology and similar fields which are becoming of increasing importance. Because of this, there have been made repeated efforts, from the time of the Lewises and Vogelaar to the present, to devise fully defined, synthetic nutrients for tissue cultures. None has, to date, proved fully satisfactory. These investigations have taken 1 of 2 general paths, the analytic and the synthetic.

A. Analytic and Supplementation Methods

CHARITY WAYMOUTH, *Royal Cancer Hospital, London*

Carrel and Baker first developed this approach. Currently Fischer in Copenhagen is its chief proponent. His procedure has been to prepare a basic non-nutritive medium made up of serum, plasma and embryo juice which has been subjected to prolonged dialysis against a Ringer glucose solution to remove all or nearly all low molecular weight components. To this he then adds supplements prepared either by controlled digestion of biologic materials or by mixing in various proportions chemically defined substances which are known to exist in effective biologic supplements.

1. *Dialysis.*—Twenty-five ml each of extract from 10 day chick

embryos, serum, or plasma, containing 0.05% heparin is dialyzed aseptically for 7–8 days at +2 C in a 2 l flask against 1.5 l Ringer solution containing 0.1% glucose (Fig. 1 in (3)). This gives a 60-fold dilution of low molecular weight components.

2. *Materials.*—Seventh to 25th passage fibroblasts from the frontal bone of 14 day chick embryos, grown in D-3.5 Carrel flasks. Control cultures (sister preparations) maintained either in standard undialyzed medium (positive control) or in dialyzed medium without supplement (negative control).

COAGULUM: 1.0 ml Tyrode solution; 0.5 ml dialyzed plasma; 3 drops dialyzed embryo extract; 0.2 ml supplement.

SUPERNATANT: 0.5 ml dialyzed serum and dialyzed embryo extract; 1.0 ml supplement.

3. *Growth estimation.*—By planimeter measurement of initial area, *A*, and final area, *B*, used to calculate the growth index

$$\frac{B - A}{A}.$$

4. *Test period.*—Negative controls showed no outgrowth and underwent rapid autolysis. Experimental cultures continued to show increase in growth index for 17 days.

5. *Supplements.*

I. Amino acid mixture after Bergmann and Niemann (1) representing the proportions found in human fibrin.

l-lysine, 2HCl	60.4 mg
l-arginine	30.8
l-tryptophane	20.0
dl-methionine	10.4
l-histidine, HCl	12.4
l-glutamic acid	56.4
l-aspartic acid	23.6
l-proline	20.4
l-cystine	5.0

The total 239.4 mg, dissolved in 100 ml of physiologic saline, gives a solution containing 22.2 mg of amino-N/100 ml. Of this, 1.2 ml taken in a total of 3.3 ml of nutrient gives an amino acid concentration of about 600 mg/l, which is a little below that determined independently by White (11) and Morgan, Morton and Parker (9) as being optimal for chick heart tissue.

II. A more complete supplement, containing a large number of substances which may be presumed to be removed from plasma and embryo extract by dialysis, was devised by Fischer *et al.* (5). It had the following composition:

NaCl	7500 mg/l
KCl	200
$CaCl_2$	200

$MgCl_2$	100
Na_2HPO_4	50
$NaHCO_3$	1000

$FeCl_2$	0.6
$CuCl_2$	0.2
$MnCl_2$	0.3
$ZnCl_2$	1
$CoCl_2$	0.01

Glucose	800
Mannose	100
Galactose	100
Inositol	20

Adenosine-3-phosphate	200
Fructose-2-phosphate	100
β-glycerophosphate	100
Inosinic acid	30

Cozymase	5
Thiamine	3
Riboflavin	0.2
Pyridoxine	0.3
Pantothenic acid	0.07
Biotin	0.007
p-aminobenzoic acid	1
Hypoxanthine	100
Nicotinic acid	0.3
Creatine	10
Choline HCl	10
Glutathione	5
Glutamine	250
Na succinate	10

Na fumarate	10
Na malate	10
Na oxaloacetate	10
Ascorbic acid	2
Methyl-naphthohydroquinone sulfate	0.005

l-lysine, 2HCl	15
dl-valine	14
l-leucine	9
dl-isoleucine	10
dl-threonine	12
dl-phenylalanine	7
l-arginine	2
dl-methionine	6
l-tryptophane	2
l-histidine HCl	5
Cystine	5

It was later concluded that most of the components of this mixture were without importance to chick osteoblasts and the following simpler solution was developed:

III. Solution V-614.

NaCl	7500 mg/l
KCl	200
$CaCl_2$	200
$MgCl_2$	100
Na_2HPO_4	50
$NaHCO_3$	1000
Glucose	2000
Fructose-2-phosphate	200
Glutamine	250
Cystine	10
Glutathione	10
l(−)lysine, 2HCl	30
l(−)histidine, HCl	10
l(−)arginine	4
dl-valine	28
l(−)leucine	18
dl-isoleucine	20
dl-threonine	24
dl-phenylalanine	14
l(−)tryptophane	4

Further study of the amino acid supplement, in conjunction with other constituents of the medium, resulted in replacing Rose's by Bergmann and Niemann's amino acid mixture (5). The earlier observation that cystine was the most important single amino acid (3) was confirmed, and it was demonstrated that it could be supplied as the free amino acid or as glutathione (cf. (12)). Other amino acids were accorded varying degrees of importance in the supplementary medium. An observation of great importance is that mixtures of amino acids in the proportions present in lactoglobulin and bovine serum albumin are much less effective supplements than peptic or tryptic digests of these proteins (5).

IV. Protein supplement. *a)* Procedure: Crystalline pepsin or trypsin was dialyzed for 24 hours. Proteins to be digested were diluted with water 1:3 and toluene was added to prevent bacterial growth. Digestion was followed by heating for 5 min at 100 C, cooling, filtration and evaporation in vacuo to remove toluene. pH was adjusted by NaOH or HCl, depending on the enzymes used. The solutions were sterilized by filtration.

b) Amino acid composition of lactoglobulin and bovine serum albumin:

Amino Acids	Lactoglobulin, Mg/20 Ml	Bovine Serum Albumin, Mg/20 Ml
Glycine	1.4	1.9
Alanine	6.2	...
Valine	5.83	6.5
Leucine	15.6	13.7
Isoleucine	8.4	2.9
Proline	4.1	5.7

Phenylalanine	3.54	6.2
Cysteine	1.11	1.11
Cystine	2.29	5.41
Methionine	3.22	0.81
Tryptophane	1.94	0.58
Arginine	2.88	6.2
Histidine	1.58	3.8
Lysine	11.4	12.4
Aspartic acid	11.4	10.6
Glutamic acid	19.5	16.9
Glutamine	1.5	1.05
Serine	5.0	4.5
Threonine	5.85	6.5
Tyrosine	3.78	5.49

c) Test conditions: Pure strain myoblasts from 9 day chick embryo in Carrel D-3.5 flasks.

COAGULUM: 0.5 ml dialyzed chicken plasma; 1.0 ml Tyrode solution; 0.1 ml supplement; 1 drop dialyzed embryo extract.

SUPERNATANT: 0.5 ml dialyzed serum; 0.1 ml supplement.

pH was adjusted with gas mixture: 8% CO_2, 12% N_2 and 80% O_2.

d) Test periods: 4–13 days.

Comment.—A qualitative examination of the amino acids, peptides and certain phosphate-containing substances of beef embryo extracts by Gordon (7) showed the presence of significant amounts of alanine, glutamic acid, glycine, glutamine, taurine and aminoethylphosphate. Tryptophane was not detected. Supplementation of dialyzed medium plus basic nutrient with synthetic aminoethylphosphate, cystine, glycine and glutamine gives excellent growth. Ehrensvärd *et al.* (2) have shown (1) that any phosphate source, together with glucose, can replace fructose-diphosphate, though aminoethylphosphate appears to be slightly more effective than inorganic phosphate; (2) that sorbose or sorbitol and rhamnose, but not ribose, can replace the fructose moiety of fructose diphosphate; (3) that alanine and glycyl peptides can replace glycine; (4) that asparagine can replace glutamine, and (5) that cystine is indispensable. Hypoxanthine in a final concentration in the medium of about 6 μg/ml stimulates mitosis. For the chick fibroblasts studied by Fischer, cystine, glycine, arginine, tryptophane and glutamine appear to be the most important supplements to the dialyzed medium.

White and Lasfargues (14) confirmed the inadequacy of Fischer's dialyzed medium for the maintenance of chick fibroblasts in vitro but observed that dilution of the medium with Tyrode solution restored some of its activity. This could be due, as White and Lasfargues suggest, to diluting out a toxic factor. A general metabolic imbalance, produced by divergence from the optimal proportions of essential nutrients, might be of more serious consequence at low than at high dilutions of the medium. The dilution effect shows that the ineffectiveness of the dialyzed medium is not wholly due to positive deficiency, and, until this important effect is more fully understood, even qualitative determinations of the nutritive value of individual supplements to these media are hazardous.

B. True Synthetic Media

PHILIP R. WHITE, *Institute for Cancer Research, Philadelphia*

In contrast to the analytical approach to the development of chemically defined nutrients carried on by Carrel and Baker and more recently by Fischer, a few investigators, notably White (11, 13) and Morgan, Morton and Parker (9), as well as Fischer (5) and Ehrensvärd *et al.* (2) have taken advantage of the great advances in our knowledge of the nutritional requirements of intact organisms, from paramecium and tetrahymena to drosophila, rats and man. This information has been used in setting up more or less arbitrary but truly synthetic, defined nutrients for animal tissues. White had previously taken a leading part with Gautheret (6) in the development, over the past 20 years, of a body of procedures and nutrients for the cultivation of excised plant tissues in vitro. The most widely used nutrient formula for plant tissues—roots, cambium, embryos, etc.—has the following constituents (10):

$Ca(NO_3)_2$	200 mg/l	$MnSO_4$	4.5 mg/l
$MgSO_4$	360.0	$ZnSO_4$	1.5
KNO_3	80.0	H_3BO_3	1.5
KCl	65.0	Glycine	3.0
Na_2SO_4	200.0	Thiamine	0.1
NaH_2PO_4	16.5	Niacin	0.5
KI	0.75	Pyridoxine	0.1
$Fe_2(SO_4)_3$	2.5	Sucrose	20 g

This is a relatively simple, clearly defined, easily duplicable nutrient. These 11 salts, 3 vitamins, 1 amino acid and carbohydrate provide all the requirements for continuous growth of a wide variety of aseptically isolated plant tissues. Such cultures have been used extensively in the study of nutritional and metabolic problems, the cultivation of viruses and other pathogenic organisms, the etiology of tumors, formative effects of chemicals, problems of morphogenesis, etc.

By application of methods similar to those employed in establishing nutrients for plant tissues, i.e., by setting up arbitrarily chosen blocks of nutrient factors (salts, vitamins, carbohydrates, amino acids, purines, etc.) and then simplifying these blocks by systematic elimination, a synthetic nutrient has been developed which will support prolonged functional survival (up to 3 months) of chick heart muscle (11, 13). Although not adequate to promote prolonged rapid growth, as does the classic embryo-juice nutrient, it is rendered complete by the addition of as little as 5% serum without embryo extract (8). This work is at the moment in a state of flux.

1. *Nutrient* (13).

Dextrose	8500 mg/l	dl-valine	130.0 mg/l
NaCl	7000	dl-isoleucine	104.0
KCl	375	dl-phenylalanine	50.0
$Ca(NO_3)_2 \cdot H_2O$	210	Glycine	100.0
$MgSO_4$	275	Cysteine HCl	1.0
$Na_2HPO_4 \cdot 12H_2O$	145	Glutathione	1.0
KH_2PO_4	26.0	Thiamine	0.1
$NaHCO_3$	550.0	Pyridoxine HCl	0.5
$Fe(NO_3)_3 \cdot 9H_2O$	1.3	Niacin	0.5
l(+) lysine HCl	156.0	Riboflavin	0.1
l(+) arginine HCl	78.0	Biotin	0.4
l(+) histidine HCl	26.0	Inositol	0.5
l(+) aspartic acid	60.0	Choline	1.0
l(−) glutamic acid	140.0	Carotene	0.1
l(−) leucine	156.0	Vitamin A	0.1
l(−) tryptophane	40.0	Ascorbic acid	0.5
l(−) proline	50.0	Calcium pantothenate	0.1
l(−) cystine	15.0	Folic acid	0.05
dl-threonine	130.0	β-alanine	0.05
dl-methionine	130.0	Phenol red	5.0

Exact directions for preparing, sterilizing and mixing the various ingredients will be found in White (11, 13).

2. *Materials.*—Heart muscle or leg muscle from 10 day chick embryos, inoculated directly into roller tubes containing 2 ml of synthetic nutrient. Mouse sarcoma (Earle's strain L) standardized on cellophane in horse serum–chick embryo-juice nutrient, then subcultured into D-3.5 Carrel flasks with 1 ml of synthetic nutrient.

3. *Evaluation.*—Survival as measured by capacity to recover when transferred to serum–embryo-juice nutrient, and maintenance of function as indicated by continuance of heart beat. Not quantitative.

4. *Test period.*—Observed until rounding of cells, autolysis or other signs of degeneration (leg muscle) or until irreversible cessation of heart beat (heart muscle). In negative controls (salt-dextrose solution) this occurs within 3–5 days, whereas in test solutions cells continue in good condition for periods up to 3 months.

Comment.—Although this nutrient is not to be looked on as a replacement for the classic embryo-juice nutrient, since it does not support prolonged and rapid multiplication of fibroblasts, it does permit functional survival for long periods, at a mitotic level more nearly approximating that in the intact organism. It would appear that the classic serum–embryo-juice nutrient simulates the conditions existing in a region of inflammation such as an unhealed wound, whereas the synthetic nutrient more nearly approximates the conditions of the intact body.

Comment by C. M. Pomerat

Most workers using tissue culture techniques agree that the development of a medium of known composition for the continuous cultivation of animal cells constitutes one of the central problems of cell biology. However, those investigators who are deeply committed to the systematic solution of the nutritional problems in the devising of a formula for a synthetic medium generally feel that their work is not sufficiently advanced to warrant incorporation in a review of established methods. Their efforts are here described as *models* of investigative methods and goals to be aimed at, which may be of great value to other workers interested in this field.

The work of Parker and his associates has been admirably summarized by J. F. Morgan in Parker's *Methods of Tissue Culture* (2d ed.; New York: Paul B. Hoeber, Inc., 1950) and therefore is not included here. A useful review of the nutrition of animal cells appeared in *Nutrition Reviews* (8: 181–182, June, 1950).

REFERENCES

1. Bergmann, M., and Niemann, C.: On blood fibrin: A contribution to the problem of protein structure, J. Biol. Chem. 115: 77, 1936.
2. Ehrensvärd, G.; Fischer, A., and Stjernholm, R.: Protein metabolism of tissue cells in vitro: 7. The chemical nature of some obligate factors of tissue cell nutrition, Acta physiol. Scandinav. 18: 218, 1949.
3. Fischer, A.: Die Bedeutung der Aminosäuren für die Gewebezellen in vitro, Acta physiol. Scandinav. 2: 143, 1941.
4. Fischer, A.: Amino-acid metabolism of tissue cells in vitro, Biochem. J. 43: 491, 1948.
5. Fischer, A.; Åstrup, T.; Ehrensvärd, G., and Oehlenschlager, V.: Growth of animal tissue cells in artificial media, Proc. Soc. Exper. Biol. & Med. 67: 40, 1948.
6. Gautheret, R. J.: *Manual technique de culture des tissus végétaux* (Paris: Masson et Cie, 1942).
7. Gordon, A. H.: An investigation of the intracellular fluid of calf embryo muscle, Biochem. J. 45: 99, 1949.
8. Jacoby, F., and Darke, S. J.: Animal tissue culture with a synthetic medium, Nature, London 161: 768, 1948.
9. Morgan, J. F.; Morton, H. J., and Parker, R. C.: Nutrition of animal cells in tissue culture: I. Initial studies on a synthetic medium, Proc. Soc. Exper. Biol. & Med. 73: 1, 1950.
10. White, P. R.: *A Handbook of Plant Tissue Culture* (Lancaster, Pa.: Jacques Cattell Press, 1943).
11. White, P. R.: Cultivation of animal tissues in vitro in nutrients of precisely known constitution, Growth 10: 231, 1946.

12. White, P. R.: The nutrition of malignant tissue in vitro, Ann. New York Acad. Sc. 49: 111, 1947.
13. White, P. R.: Prolonged survival of excised animal tissues in vitro in nutrients of known constitution, J. Cell. & Comp. Physiol. 34: 221, 1949
14. White, P. R., and Lasfargues, E.: Some effects of dilution on the nutritive value of dialyzed plasma and embryo juice, Proc. Soc. Exper. Biol. & Med. 71: 479, 1949.

II. COMMERCIAL MEDIA

DUNCAN C. HETHERINGTON, *Duke University*

From the time of its organization in 1946 following the Hershey, Pa., conference, the Tissue Culture Commission, since 1949 known as the Tissue Culture Association, has had for its purpose the aim "to foster the collection and dissemination of information concerning the maintenance and experimental use of tissue cells in vitro and the evaluation and development of procedures therefor." In other words, it is a service organization. One of its first projects was to investigate the kinds of media needed by workers in the field and to examine the possibility of preparing these ingredients at some central laboratory for distribution.

It was realized that in most laboratories, and especially in small ones where technical help is not abundant, much of the load of routine devoted to the preparation of media might be decreased enormously by the availability of commercially prepared, standardized, basic media constituents. It was felt that this would encourage a more rapid development and wider use of the tissue culture technique and, as a corollary, give greater time to the investigator for productive work.

As a preliminary step in determining what media were considered "basic," a questionnaire was sent to the members requesting information as to their culture problems and as to what media they would be most likely to require, if such were to be made commercially. Tabulation of returns listed embryo extract, chicken plasma, human placental cord serum, horse serum and serum ultrafiltrate as well as the more commonly used buffered saline solutions and thrice-distilled water.

After due consideration it was agreed that probably serum, plasma and embryo extract would suffer least denaturation and remain more stable during storage and shipment if they were prepared by lyophilization. The work of Hetherington (2) and Craig (3, 4) and Gey and Gey (1) indicated that frozen-dried media had certain advantages over freshly prepared materials. Accordingly, very careful specifications covering all phases of preparation of the proposed media—from selection of virus- and disease-free sources of biologic supply to the details of processing—were drawn up as a guide for commercial production. In addition a testing laboratory, sponsored by the Association and made possible by generous grants from the Committee on Growth acting for the American Cancer Society, was set up to counsel the producers on technical problems and to test and to certify the commercial products as satisfactory for growth of tissues in vitro.*

The Difco Laboratories, 920 Henry St., Detroit, Mich., and the Microbiological Associates, Inc., of Coral Gables, Fla., have cooperated in every way to produce media of the highest quality and are ready to increase the variety of products as the occasion demands.

It is not known at this time how many investigators use the commercial media in their routine culture work or how fully these media compete with freshly prepared materials. Some reports, however, indicate that a few researchers rely on commercial media almost entirely and that they have found them to be satisfactory and well suited to their problems. The most consistent criticism has been related to a slight delay in clotting time of the frozen-dried plasma after reconstitution. This slight annoyance has, however, been largely corrected by bringing the dried product to volume with distilled water saturated with CO_2 or by allowing the plasma, reconstituted with distilled water only, to equilibrate in an atmosphere of 5% CO_2 before use. This treatment serves a double purpose of correcting clotting time and lowering the pH to the most desirable level.

Some concern has been voiced over the relatively high cost of the commercial media. This factor will certainly decrease in proportion to increasing demand as more investigators become aware of their value. Some laboratories have already intimated that, even with the prevailing prices, their productiveness in research due to time saving in routine has outweighed this factor.

REFERENCES

1. Gey, G. O., and Gey, M. K.: The maintenance of normal cells and tumor cells in continuous culture: I. Preliminary report; cultivation of mesoblastic tumors and normal tissues and notes on methods of cultivation, Am. J. Cancer 27: 45–76, 1936 (footnote 7, p. 57).
2. Hetherington, D. C.: Frozen-dried serum as a medium constituent for tissue cultures, Proc. Soc. Exper. Biol. & Med. 57: 196–197, 1944.
3. Hetherington, D. C., and Craig, J. S.: The effect of frozen-dried embryo juice on tissue cultures, Proc. Soc. Exper. Biol. & Med. 42: 831–834, 1939.
4. Hetherington, D. C., and Craig, J. S.: Tissue culture growth stimulants from ground frozen-dried chick embryos, Proc. Soc. Exper. Biol. & Med. 44: 282–285, 1940.

---

* Questions regarding testing procedures may be directed to Keith R. Porter, Rockefeller Institute for Medical Research, 66th St. and York Ave., New York 21, who directed the project.

TISSUE CULTURE PROCEDURES IN MEDICAL INSTALLATIONS

MARGARET R. MURRAY, *Columbia University*

A. Sources and Handling of Material

A RICH AND almost untapped vein of material for medical research runs through the surgical service of a large hospital. It is unequaled as a source of normal and neoplastic human tissues which with the aid of steadily developing culture techniques can be studied directly in the living form and at the cellular level. The uses and advantages of cultured human tissues for medical investigations are many, and the detailed methods of handling are correspondingly varied. One aspect, not to be despised, is the economics of obtaining sterile specimens for tissue culture in the most viable state, with the least investment of time and inconvenience on the part of the laboratory staff and of the operating team. The following system has been found workable and not too burdensome when added to an operative routine already well saturated as to detail.

The co-operating surgeon is apprised of the type of specimen desired. He arranges to have the laboratory informed of operations that may provide such material, preferably the day before they take place. The operating room maintains a stock of sterile Petri dishes 5 or 10 cm in diameter, prepared for autoclaving in such a way that they can be placed on the instrument table to be handled by the sterile nurse. The surgeon, who is acquainted with the general qualifications for good tissue culture material, selects it at a convenient moment and puts it into the Petri dish. An assistant calls the laboratory, which sends for the specimen. This routine provides a means of getting excised tissues into culture with a minimum of delay and exposure; it is simple and avoids many accident hazards, consequently we use it whether speed is important or not. For most favorable results the specimen used for tissue culture should be fresh, aseptic, moist (not subjected to the drying effects of air), cellular (i.e., meaty rather than fibrous or mucinous), well vascularized, non-necrotic; subjected to a minimum of trauma of handling, selected from a site at least 1 cm from any cauterized area. Wrapping of the tissue in moist gauze is undesirable because cotton fibers adhere to it and find their way into the cultures.

Placental serum is a valuable ingredient of tissue culture media which is available in most hospitals with a minimum of inconvenience. A cylindrical glass tube 3–4 cm in diameter and 15–20 cm long is a good receptacle for placental blood. If this is provided with a glass cover which fits down over it loosely, the whole may be doubly wrapped in cloth and autoclaved. The tube may then be handled by a sterile nurse in the delivery room. Blood is collected only from Wassermann-negative patients having uncomplicated deliveries. After the baby is delivered and the umbilical cord cut, the maternal end of the cord may be unclamped and allowed to bleed into the tube until the blood clots or the placenta is delivered. The tissue culture laboratory is then called and the tube is collected. The clot is allowed to harden at room temperature for about 1 hr, then is gently loosened from the walls of the tube with a blunt glass rod, and the covered tube is refrigerated at about 4 C over night. During this time syneresis proceeds and the cells settle to the bottom with the clot. The supernatant serum should be drawn off as soon thereafter as possible, to prevent hemolysis. It is then stored at 4 C for at least 2 weeks; by the end of this period it will be found to be sterile.

B. Double Cover Slip Culture Technique

The double cover slip, lying-drop mode of cultivation originated by Maximow is the method of choice for close observation of cells, either living or specially stained, at high magnification. Cultures so handled are especially suited to photography, either still or by time lapse over periods of several hours; to experimental treatment with biologically active compounds, to which the tissue response can be observed minutely and repeatedly; to cytologic observations of all sorts, and to studies of differentiation and histogenesis.

Supplies.—Some special supplies and equipment appropriate for this method follow:

1. Noncorrosive glass cover slips (Gold Seal): (*a*) round, $^7/_8$ in. D no. 1 thickness; (*b*) square, 40 × 40 mm—no. 3 thickness for ordinary use, no. 1 for observations with oil immersion.
2. Glass depression slides 75 × 45 × 5–7 mm, with ground and polished concavity 35 mm in diameter.
3. Small Coplin jars designed to hold cover slips in vertical position (Columbia staining dishes).
4. Instruments: flat-tipped cover slip forceps; screw-tipped needle holders to contain no. 9 steel sewing needles and 2 in. lengths of narrow gauge platinum wire hammered flat at the tip to form spreaders; small glass rods pulled thin at tips and bent, to form spreaders.

5. Large Petri dishes, 15 cm diameter.
6. Black glass or plastic (heat resistant) block about 5 cm square and 1 cm high.
7. Wooden slide racks 15 × 9 × 5 cm for supporting groups of 6 slides one above the other, with about 1 cm air space between.
8. Petrolatum for sticking slides to cover slips; petrolatum-paraffin mixture for sealing (1 pt petrolatum to 5 pt 50° paraffin).

For microscopic observations, the combination of 8 mm apochromat objectives (20×) with 15× Kompensating or Periplan oculars, a lowered aplanat condenser and concave mirror tilted slightly off center gives excellent resolution for such transparent objects as living cells. It suffices for all routine observations, and a good deal of photography, and can be used with no. 3 square cover slips, whose sturdiness is essential for routine handling. When higher magnification is desired a 3 mm apochromat oil immersion objective is recommended, coupled with a no. 1 square cover slip.

PROCEDURE

It is assumed that sterile materials and aseptic methods of handling are used throughout.

In the large Petri dishes, which contain a strip of black photographic paper on a disk of white filter paper, 2 square cover slips are placed. A very small drop of BSS (*vide infra;* also (9)) is put in the middle of each of these, and with the aid of the flat-tipped forceps a small round cover slip is centered on this so that it adheres without slipping and without air bubbles. About 0.1 cc (2 drops) of feeding solution is dropped in the middle of this and the explants (1, 2 or 3) are put into it. An equal quantity of plasma-serum mixture is then dropped on, and the whole is spread over the entire surface of the round cover slip; the explants are then oriented. A depression slide with 3 small dabs of petrolatum spaced around its well is inverted over the cover slip; the culture is then lifted gently from the Petri dish and left undisturbed until it clots. Shortly thereafter it is sealed with the petrolatum-paraffin mixture, placed in the rack in the original position, cover slip down, and incubated. For both avian and mammalian tissues 35–36 C is a satisfactory temperature.

Three times a week the cultures are washed and refed. To do this the sealing mixture, while still warm, is loosened along its outer edge with a scalpel or razor blade, grasped with the fingers and pulled off. The large cover slip is placed buttered side up on the black glass block, and with the aid of the needle and the flat-tipped forceps the small cover slip bearing the culture is pried loose and dropped into a small Coplin jar filled with BSS at a pH of about 7.3. Four cultures can be washed at once. The washing period may be from 5 to 30 min, depending on age of culture and amount of outgrowth. The round cover slip is then withdrawn slowly from the washing dish, to drain off the saline as completely as possible, and is centered on a fresh square cover slip in a Petri dish. One drop of feeding solution is delivered from above, and a fresh depression slide is inverted over the culture.

For the cultivation of both normal and neoplastic tissues by this method, the following ingredients may be combined in varying proportions: chicken plasma, 50% saline extract of 9 day chick embryos (EE), human placental serum, beef serum ultrafiltrate and a buffered saline solution (Simms X-7) referred to as BSS. A good general feeding formula for human tissues consists of: placental serum 3 parts, chick EE 1 part and serum ultrafiltrate 1 part. For explantation, chicken plasma is substituted for the serum ultrafiltrate (above), and 2 separate mixtures are made, to be combined in equal amounts on the cover slip:

Feeding Solution	
Placental serum	3 parts
Chick EE	2 parts

Plasma Mixture	
Placental serum	3 parts
Chicken plasma	2 parts

Rat tissues also thrive on this formula; no murine components seem to be required. For chicken tissues, however, it is necessary to reduce the amount of human serum and increase the amounts of chick EE and ultrafiltrate. For myelin-producing cultures of embryonic chick spinal ganglia a favorable feeding formula is composed of equal parts serum, EE and ultrafiltrate. The ganglia are explanted into a rather thin clot:

Feeding Solution	
Chick EE Placental serum	equal parts

Plasma Mixture	
Chicken plasma	7 parts
Serum ultrafiltrate	7 parts
Placental serum	6 parts

The medium formulas recommended here are rich as compared with those used in flask and roller tube cultures, which are designed for more rapid cell propagation. But this discrepancy is overbalanced by the smaller ratio of medium volume to cell population volume and by the higher ratio of placental serum which presumably favors differentiation. According to Gaillard and to Fell, extracts from late embryos favor histogenesis; it might be assumed that fetal-placental serum taken at term exerts similar effects. It should also be noted that, from the first washing on, there is a considerable residue of BSS in the interstices of the clot at the time the new feeding solution is dropped on.

Probably the balance of the ingredients in any formula is more important than the absolute amount of any component, and innumerable specific variations of the stock formulas are used successfully. In general, serum ultrafiltrate inhibits or prevents the accumulation of lipoid droplets in cells; a reduction in the amount of EE and its replacement by BSS decreases cell multiplication; placental or adult serum favors differentiation. Epithelium, macrophages, and some nerve tissues, require a relatively low content of EE; muscle requires high EE, up to 40% of the formula; fibroblasts do well with moderate amounts of EE, are inhibited by large amounts of placental serum.

The most favorable size for explants varies from tissue to tissue. For nerve and epithelial tissues (normal or neoplastic) many small explants are better than a few large ones. For mesoblastic tissues, muscle especially, the reverse is true; 2 strips of skeletal muscle 1 × 1 × 3 mm produce a favorable cover slip culture. In epithelial tissues, adenomas, carcinomas and sympathicoblastomas, it is well to persuade this friable material to break apart so that very small nodules will separate themselves from the stromal tissues. This "soup," reinforced by a few small explants of less than 1 cu mm, responds well in culture.

Comment.—The over-all objective of the double cover slip method is to provide an environment in which cell multiplication goes on at a relatively low level (though it is not excluded altogether) and cellular differentiation is favored. For these ends, the culture should remain in its original site, without transferral. It is possible to maintain human tumor cultures in this way for at least a month and differentiating normal tissues (such as spinal ganglia) for several months. It has been found possible to carry adult rat skeletal muscle in such a preparation for a year and a half. In all these instances the culture period was voluntarily brought to an end, with the tissue in healthy condition. During these long periods free from mechanical disturbance, not only may functional and morphologic specialization occur but characteristic growth patterns may be revealed, and interrelations between types of cells, such as neurone and supporting cell, may be established.

REFERENCES

1. Gaillard, P. J.: Die ontogenetische Aenderung in der Zusammensetzung der Körperflüssigkeiten in Hinblick auf das Wachstum und die Differenzierung von darin gezüchteten Geweben, Acta brev. Neerland. 2: 16–19, 1932.
2. Gaillard, P. J.: Developmental changes in the composition of the body fluids in relation to growth and differentiation of tissue cultures, Protoplasma 23: 145–174, 1935.

3. Murray, M. R., and Stout, A. P.: The glomus tumor: Investigation of its distribution and behavior, and the identity of its "epithelioid" cell, Am. J. Path. 18: 183–203, 1942.
4. Murray, M. R., and Stout, A. P.: Neuroepithelioma of the radial nerve with a study of its behavior in vitro, Rev. canad. de biol. 1: 651–659, 1942.
5. Murray, M. R., and Stout, A. P.: Distinctive characteristics of the sympathicoblastoma cultivated in vitro: A method for prompt diagnosis, Am. J. Path. 23: 429–442, 1942.
6. Murray, M. R., and Stout, A. P.: A sympathetic ganglioneuroma cultivated in vitro, Cancer 1: 242–247, 1948.
7. Peterson, E. R.: Production of myelin sheaths in vitro by embryonic spinal ganglion cells, Anat. Rec. 106: 66, 1950 (abst.).
8. Pogogeff, I. A., and Murray, M. R.: Form and behavior of adult mammalian skeletal muscle in vitro, Anat. Rec. 95: 321–336, 1946.
9. Simms, H. S., and Sanders, M.: Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses, Arch. Path. 33: 619–635, 1942.

GENERAL METHODS FOR QUANTITATIVE STUDIES ON CELLS IN TISSUE CULTURE

Method for Growth of Single Isolated Tissue Cells and Development of Pure Cell Strains

WILTON R. EARLE, *National Cancer Institute*

SUCCESSFUL ESTABLISHMENT of a pure strain from an isolated cell has been achieved using the L strain of mouse cells (1). This result was developed from the concept that even the best of culture media are actually poorly suited for the growth of tissue cells in vitro until the medium has been altered by the metabolic activity of living cells. The key features of the method for the initiation of growth from a single isolated cell are (1) cultivation within the confines of a capillary tube so as to restrict diffusion of the medium around the cell and (2) the use of culture medium derived from a rapidly growing large culture.

PROCEDURE

The isolation of a single cell can be made from a Carrel flask constantly gassed with 5% CO_2 in air to control the pH. From 2.8 mm Pyrex tubing, micropipets are drawn to an internal diameter of approximately 0.1 mm; their total length averages 125 mm, of which about 25 mm at 1 end is unconstricted and plugged with cotton. Such micropipets are joined to a cotton-packed bacteria filter tube and to a mouth piece by hemocytometer tubing. For use, micropipets are flushed with chicken plasma to form a thin lining film. Single cells are isolated free-hand under a dissecting microscope equipped with 10× wide field oculars and 4× objectives. One to several cells can thus be collected. The capillary tubes are broken in lengths of approximately 100 mm and sealed off by means of a molten glass bead on an electrically heated platinum wire. After selection of well isolated, healthy single cells with a microscope system at approximately 500×, the capillaries are sterilized by immersion in chloroform for 15 min. Sections of capillary tubes 5 mm long containing desirable isolated cells are cut with the blunt edge of a sterile Bard Parker knife blade no. 23. The tubes are embedded in a mixture of 0.5 ml chicken plasma and 0.5 ml conditioned culture fluid in D-3.5 Carrel flasks.

The "conditioned" culture fluid was drawn from large plasma cultures showing vigorous growth after 18–24 hr at 37.5 C. With such "conditioned" medium a culture consisting of some 100 cells was developed within 11 days from an isolated single cell. This pure cell strain has been maintained for more than 2 years in a completely heterologous culture medium consisting of dilute horse serum and chick embryo extract. It has preserved the original morphologic characteristics of the L cells, even to the production of numerous giant cells.

No other cell strain has yet been grown from a single cell. Data on hand suggest, however, that the procedure may have general applicability when certain interfering variabilities in the composition of the culture medium have been eliminated.

REFERENCE

1. Sanford, K. K.; Earle, W. R., and Likely, G. D.: The growth in vitro of single isolated tissue cells, J. Nat. Canc. Inst. 9: 229–246, 1948.

Method for Growth of Cells on Perforated Cellophane

WILTON R. EARLE, *National Cancer Institute*

Although fibrin clots are exceedingly valuable for obtaining luxuriant cell growth in vitro, the mechanism of action of the plasma clot remains unexplained. For many types of experiments plasma clots are troublesome because they may become cloudy or liquefied; they hinder the collection of cells for quantitative chemical studies, and the clot itself is of undefined chemical composition. Excellent cell proliferation has been obtained under a sheet of perforated cellophane with use of a horse serum-chick embryo extract fluid medium (3). Unperforated cellophane was found less suitable. Variation of the perforation design was of only minor significance in the growth of cells of strain L mouse sarcoma and of strain 224 (2).

PROCEDURE

DuPont cellophane PT62, thickness 450, with Beckley Perforation design OC, hole diameter 0.023 in., diagonal pattern 45° centers, 324 holes/sq in., $13^1/_2$% open area, is used. The cellophane is washed in three 10 min changes each of acetone, ether, alcohol and distilled water. Sterilization is by autoclaving, but this should not be done more than once. In D-3.5 Carrel flasks, 1.0 ml of a mixture of 40% horse serum, 20% embryo-juice extract (1:1) and 40% Earle's saline is added and allowed to soak in the cellophane for 1–3 min. Cell clumps are introduced under the cellophane sheet with a platinum spatula.

Satisfactory cultures can be obtained by adapting cellophane to double cover slip slide preparations. These allow examination at high magnification of cells growing in absence of plasma clots (4).

A 30 mm round cover slip is attached to a 48 mm square cover slip by a small drop of fluid culture medium. Three drops of fluid medium consisting of horse serum and chick embryo extract is placed on the round cover slip. The explant is transferred to this and covered by a disk of perforated cellophane 28 mm in diameter. The large square cover slip is sealed onto a Maximow slide with paraffin.

Blake-type culture bottles of 1 l capacity, each containing a strip of perforated cellophane 80 mm wide and 200 mm long, permit excellent growth of chicken fibroblasts and of the strain L mouse cells (1). The best method of seeding consists in introducing a suspension of cells and small cell clumps, although good results can be obtained from use of discrete tissue explants. Fluid medium consists of 40% horse serum, 20% 1:1 chick embryo extract and 40% Earle's balanced saline solution.

Comment.—Although the role of the cellophane is imperfectly understood, it possibly (1) prevents mechanical disturbance of cells, (2) allows cells to accumulate a zone of modified or "adapted" culture medium around themselves, and (3) proves a more favorable surface for the attachment of cells than does the glass surface of culture vessels.

REFERENCES

1. Earle, W. R.; Evans, V. J., and Schilling, E. L.: Extension of cellophane substrate procedure to growth of in vitro cultures of large areas, J. Nat. Cancer Inst. 10: 943–967, 1950.
2. Earle, W. R., *et al.:* Influence of perforation design on growth of tissue cells under perforated cellophane sheets in vitro, J. Nat. Cancer Inst., 10: 291–295, 1949.
3. Evans, V. J., and Earle, W. R.: The use of perforated cellophane for the growth of cells in tissue culture, J. Nat. Cancer Inst. 8: 103–119, 1947.
4. Schilling, E. L.; Earle, W. R., and Evans, V. J.: Use of perforated cellophane substrate in slide preparation tissue cultures, J. Nat. Cancer Inst. 10: 883–891, 1950.

Measurement of Proliferation of Tissue Cultures by Enumeration of Cell Nuclei

KATHERINE K. SANFORD, *National Cancer Institute*

The isolation of nuclei by means of citric acid and centrifugation allows their enumeration in a hemocytometer chamber following light staining with crystal violet.

Counts made on an aliquot of the original cell inoculum suspension and samples from a series of replicate cultures permit the plotting of growth curves in tissue culture experiments. This method has been developed with the L strain of mouse cells but appears to be applicable to other cell types (1).

PROCEDURE

Approximately 14 ml of 0.1M citric acid, warmed to 37 C, is added to each culture growing in 2 ml of culture fluid in a T-12 flask. The culture is incubated at 37 C for 1 hr, then shaken vigorously by hand. The nuclei are thrown down by centrifugation for 20 min at 1100 rpm. After approximately 12 ml of supernatant is withdrawn by long tip pipet and discarded, 1 ml of 0.1% crystal violet is added to each flask. The flask is then shaken by hand and the contents are transferred quantitatively, with 3–4 citric acid washes, to a 10 ml graduated cylinder. Before the transfer the outer lip of each flask is coated with yellow petrolatum to prevent dripping and consequent loss of fluid during transfer of the contents. After the cylinders are centrifuged at 1800 rpm for 20 min the supernatant is withdrawn by long tip pipet so that a volume equal to $^1/_2$ the final volume is left in each cylinder. To this volume an equal volume of 0.5% Methocel (4000 cps) is added. The final volume of suspension is roughly approximated to yield about 300,000 nuclei/ml. The graduated cylinder is stoppered and shaken to disperse the packed nuclei. While the nuclei are still uniformly dispersed, the suspension is transferred immediately to a 30 ml beaker which is kept agitated during withdrawal of the contents into the special counting pipet. If counting is to be delayed, the pipets are sealed and stored at 5 C. At the time of counting, the pipets are clamped in the Bryan-Garrey rotor and rotated for at least 15 min to establish and maintain a uniform dispersion of nuclei before samples are taken. Before the hemocytometer chamber is charged, the contents of the tip of the counting pipet are discarded. A sample of the suspension is then discharged into 1 chamber of a hemocytometer positioned on the microscope stage.

Each suspension of nuclei is sampled and counted. The operator fills 3 hemocytometer chambers and counts the nuclei in the nine 1 mm squares or hemocytometer units in each. Since the nuclei in a total of 27 squares are counted, a volume of 2.7 mm^3 is counted on each suspension. The number of nuclei/ml is calculated from the following formula:

$$\frac{\text{total no. of nuclei counted} \times \text{total vol. of suspension in mm}^3}{\text{vol. of sample counted in mm}^3} = \text{nuclei/ml}$$

Example: If the operator counts a total of 832 nuclei in 27 hemocytometer squares or a volume of 2.7 mm³ of a suspension that has been diluted to 10 ml or 10,000 mm³, the number of nuclei/ml is calculated as follows:

$$\frac{832 \times 10{,}000}{2.7} = 308{,}148 \text{ nuclei/ml}$$

It can be shown statistically that the over-all error in the preparation and planting of cultures from typical replicate cell suspensions and in the enumerations of their nuclei allows a routine over-all variability of less than 10%, with 95% probability of accuracy. Higher accuracy can be obtained with more arduous sampling.

REFERENCE

1. Sanford, K. K., *et al.:* The measurement of proliferation of tissue cultures by enumeration of cell nuclei, in press.

Preparation and Handling of Replicate Tissue Cultures for Quantitative Studies

VIRGINIA J. EVANS, *National Cancer Institute*

The effect of various agents on cell proliferation and disintegration can be measured accurately with replicate tissue cultures planted in large numbers and grown with minimal loss of cells at each renewal of the culture fluid. Genetic homogeneity is assured with the use of a cell strain originating from a single cell. Cultures are planted on a cellophane substrate and fed with a fluid medium to avoid the disadvantages of plasma clots. Cell populations are measured by the technique of enumerating cell nuclei (see p. 219). Procedures used assure adequacy of sampling, protection of cells against changes in pH, especially during stirring of the suspensions, and no loss of cells in the fluid renewals (1).

PROCEDURE

a) Preparation of cell suspensions.—In a medium consisting of 40% horse serum, 20% (1:1) embryo extract and 40% balanced saline solution, parent cultures are grown for 10–14 days on cellophane in a quantity adequate to provide the necessary amount of cells for a desired experiment. A suitable density for transfer is represented by a count in the parent culture of approximately 3,000,000 nuclei/ml of culture fluid. Cells are scraped off both sides of the cellophane sheet and the floor of the glass container with a platinum rake (Fig. 1, *A* and *B*). Pooled cell suspension is centrifuged at 1100 rpm for 15 min. The supernatant is replaced

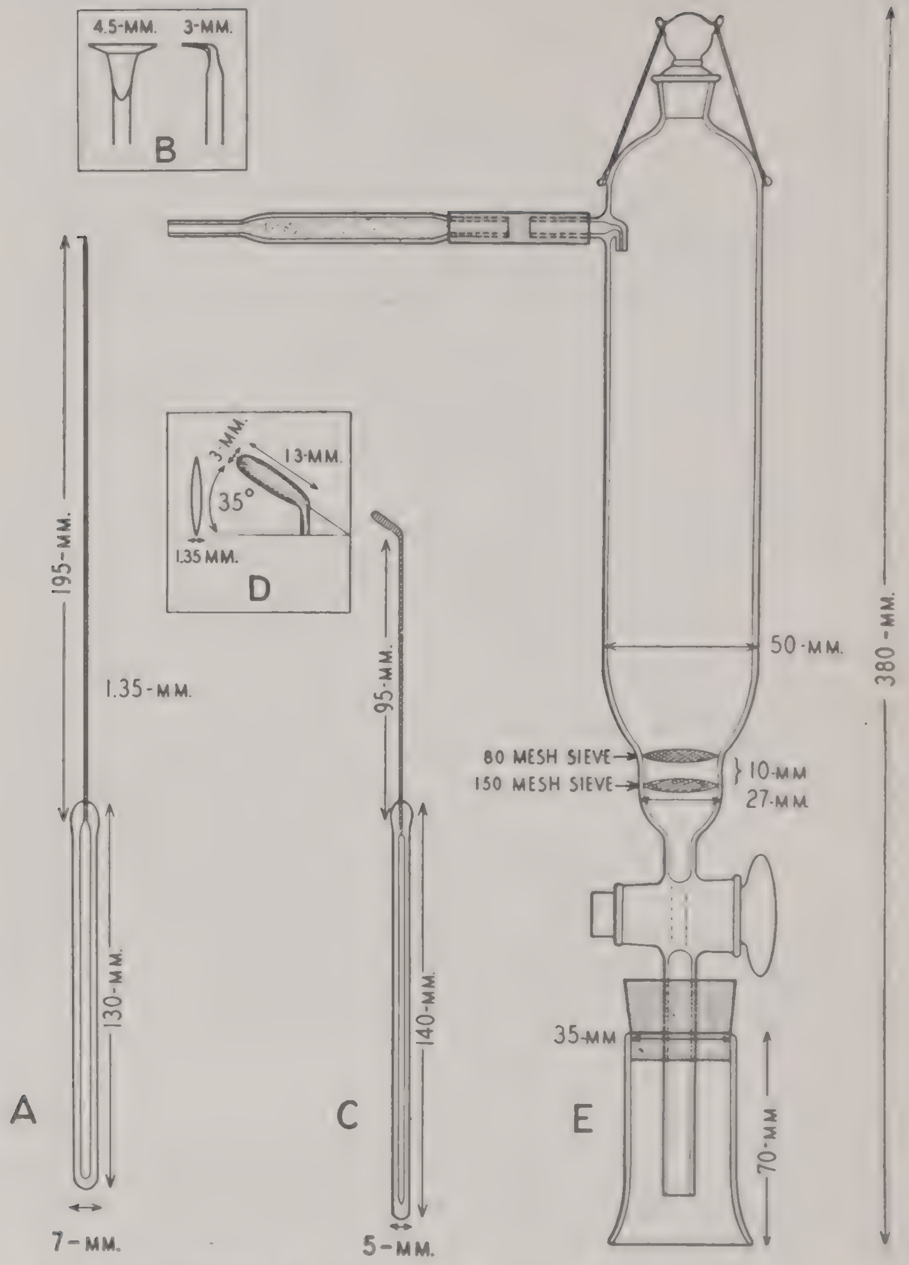
4.5-MM.
3-MM.
B
195-MM.
1.35-MM.
130-MM.
A
7-MM.
3-MM.
13-MM.
35°
1.35 MM.
D
95-MM.
140-MM.
C
5-MM.
50-MM.
380-MM.
80 MESH SIEVE
150 MESH SIEVE
10-MM
27-MM
35-MM
70-MM
E

FIG. 1. Equipment for preparing cell suspension. *A*, rake for scraping cells from cellophane and glass substrate of T-60 flasks; *B*, detail of rake tip; *C*, spatula for scraping cells from cellophane and glass substrate of T-12 flasks; *D*, detail of spatula tip; *E*, sieve unit for sieving cell suspensions.

by a volume of fluid calculated to yield a cell concentration higher than that finally desired. The suspension is transferred by a 100 ml pipet and allowed to flow through the sieve assembly (Fig. 1, *E*).

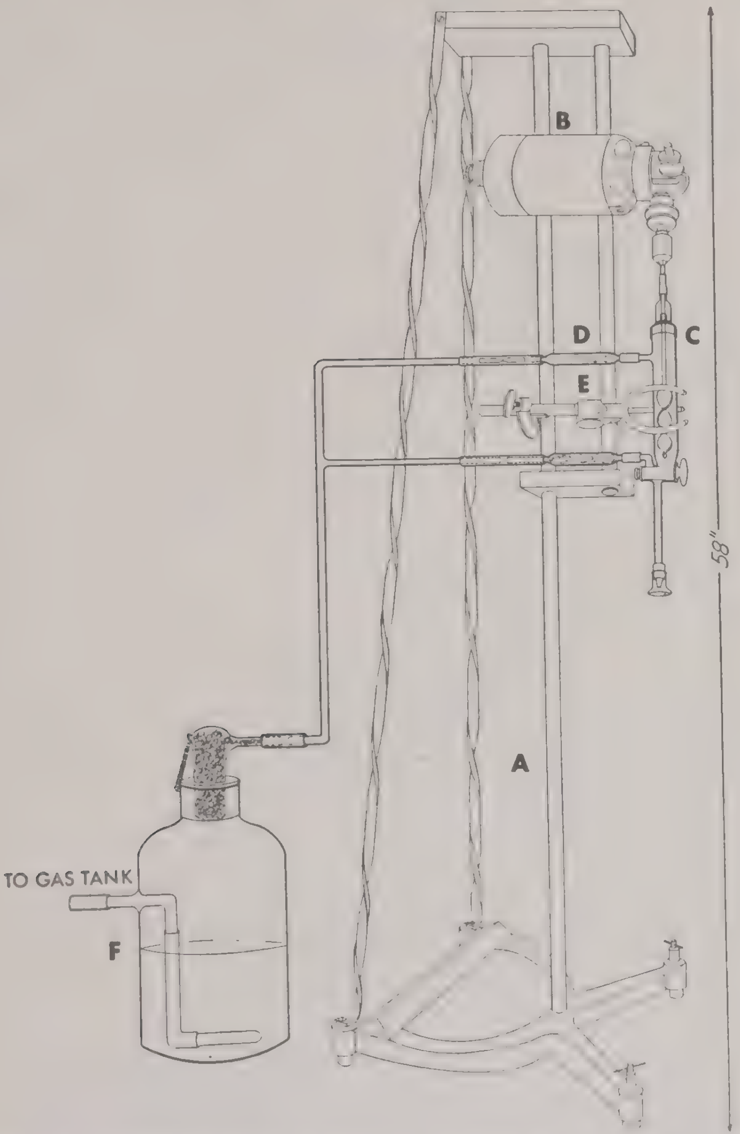

FIG. 2.—Arrangement of apparatus for stirring and dispensing cell suspensions. *A*, support stand; *B*, electric stirrer; *C*, reservoir assembly; *D*, gas trap; *E*, versatile clamp; *F*, gas humidifier. A 3-way stopcock is shown below versatile clamp.

b) Stirring and gassing of cell suspension.—The total volume of the sieved suspension is measured and introduced into a reservoir where it is kept stirred at 450 rpm during sampling and for at least 15 min before sampling. The assembly (Fig. 2) permits the entrance of gas, saturated with water vapor, to adjust and maintain the pH at approximately 7.3.

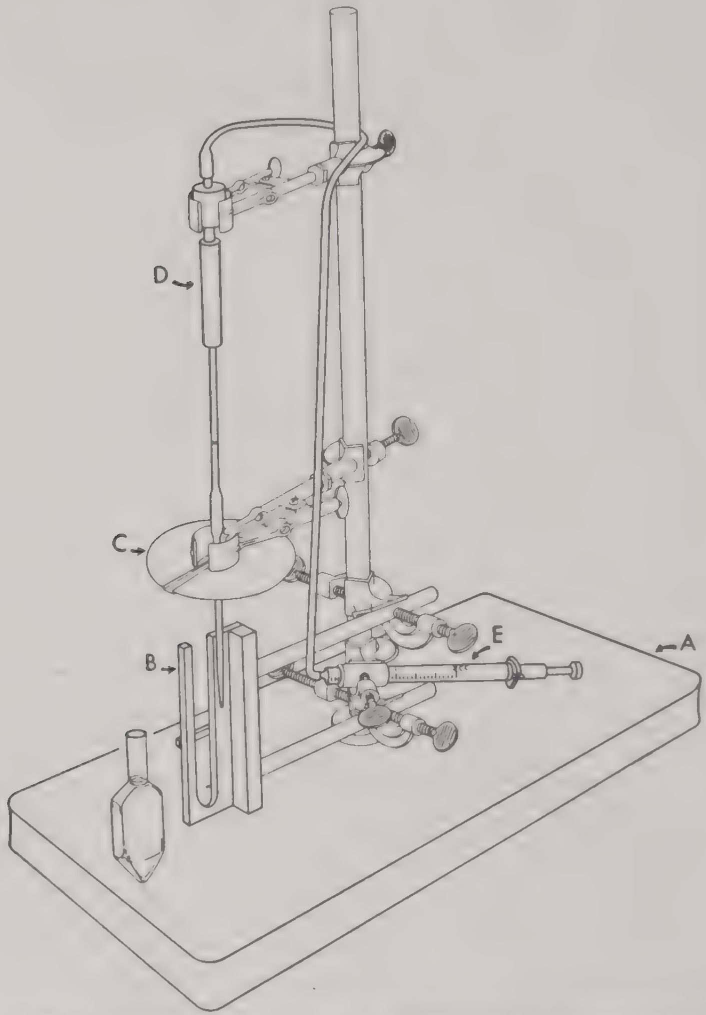

Fig. 3.—Fluid removal assembly for withdrawing supernatant fluid from T-12 flasks. *A*, rectangular porcelain support stand; *B*, flask-centering frame; *C*, pipet positioning clamp and shield; *D*, rubber coupling for pipet; *E*, 2 ml syringe.

c) *Standardizing of cell suspension.*—By means of a 3-way stopcock a 1 ml sample is discharged from the delivery tip of the reservoir by a jet of gas mixture at about 2 lb/sq in. The number of cells in the sample as determined by nuclear count is used in calculating the dilution of the stirred suspension necessary to yield the desired number of cells for inoculation of culture flasks.

d) Inoculation of culture flasks.—A 3.8 cm square of OC perforated cellophane in a T-12 culture flask is wet with 1.5 ml of the fraction of culture medium not used in suspending the cells. Each flask is inoculated with 1 ml of stirred suspension ejected from the reservoir delivery tip by the gas. The gas mixture is allowed to flow for 30 sec to replace the air by the 5% CO_2 in the gas mixture. Flasks are quickly closed with size 00 rubber stoppers and placed in the incubator.

e) Renewal of culture fluid.—For this procedure, made 3 times weekly, flasks are centrifuged at 1100 rpm for 20 min, then placed in upright in a suitable rack. An assembly designed to assure the removal of 1 ml of the fluid in the axis of the cone-shaped tip of the flask is shown in Figure 3.

After removal of the fluid, the packed cells are resuspended by vigorous tapping of the flask, the sheet of cellophane is realined on the glass and 1 ml of culture fluid is added. The flasks are gassed before stoppering.

REFERENCE

1. Evans, V. J., *et al.:* The preparation and handling of replicate tissue cultures for quantitative studies, in press.

Measurement of Growth by Nucleic Acid Determinations

CHARITY WAYMOUTH, *Royal Cancer Hospital, London*

With the aim of determining the "total number of cells present, together with some estimate of their size," Willmer (31) applied the method of Berenblum, Chain and Heatley (2) for nucleoprotein phosphorus (NPP) to roller tube tissue cultures. The technique was subsequently applied, with little modification, by Davidson and Waymouth (7, 8, 10, 11) and has been used in the testing laboratory of the Tissue Culture Association for assaying commercial media. Pentose nucleoproteins are now known to represent a high proportion of the total cell nucleoprotein (4, 9, 29), and the average amount of desoxyribonucleoprotein phosphorus per cell nucleus is sufficiently nearly constant (±10%) to permit its use as a reference standard in growth measurements (5, 6). Determinations of both pentose and desoxypentose nucleic acids have been undertaken (12, 15), with adaptation of the methods of Schmidt and Thannhauser (24) and of Schneider (25, 26) for separation.

Methods for determination of μg quantities of nucleic acids have been described by Steele, Sfortunato and Ottolenghi (28) and by Dackermann and Patterson (3) and, although they have not been applied to tissue cultures, are given here because they are applicable to the amounts of tissue commonly available in tissue cultures.

I. ESTIMATION OF NPP IN ROLLER TUBE TISSUE CULTURES

The method of Davidson and Waymouth (7) follows Berenblum, Chain and Heatley (1, 2).

PROCEDURE

a) Reagents.—(1) Ethanol-chloroform, 3:1. (2) 0.1N, HCl. (3) Perchloric acid, 60% (w/w). (4) 10N H_2SO_4 (approx.): 28% conc. H_2SO_4. (5) N H_2SO_4 (approx.): solution (4) diluted 10 times. (6) 5% ammonium molybdate; keep in a bottle coated inside with paraffin wax. (7) Stock solution of stannous chloride: 10 g of $SnCl_2$ in 25 ml conc. HCl. (8) Dilute stannous chloride: solution (7) diluted 1:200 with N H_2SO_4 as required. (9) Isobutanol. (10) Ethanol.

b) Tissue culture method.—This can be applied to roller tube cultures containing amounts of tissue of the order of 2–15 mg in a plasma or fibrin coagulum. The fluid medium is discarded and the cultures are washed briefly with physiologic saline.

c) Extraction of lipid and acid-soluble phosphorus.—The tissue and coagulum are extracted for 2 successive periods of 2 hr with warm ethanol-chloroform under a small reflux condenser. Acid-soluble P is removed by extraction for 3 hr with 0.1N HCl in the cold, with continuous agitation.

d) Estimation of phosphorus.—Useful range, 0.1–10 μg P. The contents of the tube are washed with 0.2 ml of 60% perchloric acid, cooled and transferred with the aid of 0.8 ml water to a micro separating vessel. The micro separators of Davidson and Waymouth (7) and of Kirk and Danielson (17) have the advantage over the original mixing vessel of Berenblum and Chain (1) that several separators can be set up when a series of estimations is to be made. Next, 0.25 ml of 5% ammonium molybdate and 1.0 ml of isobutanol are added, the mixture is shaken thoroughly and the aqueous layer discarded. The alcoholic solution is washed by shaking with 2 successive portions of 1.0 ml of N H_2SO_4. Then, 1.5 ml of dilute stannous chloride solution is added, the mixture shaken for 30 sec and aqueous layer rapidly discarded and the blue solution transferred to a small tube graduated at 1 or 2 ml and made up to the required volume with ethyl alcohol. The colour intensity is measured with an absorptiometer or spectrophotometer with microcuvets.

II. ESTIMATION OF PENTOSE AND DESOXYPENTOSE NUCLEIC ACID FRACTIONS

A. METHOD OF DAVIDSON, LESLIE AND WAYMOUTH (12)

A modification of the Schmidt and Thannhauser (24) separation is used. As an alternative to measurements of RNAP and DNAP, the sugar components can be measured by the orcinol and diphenylamine reactions for pentose and desoxypentose, respectively.

a) Reagents.—(1) Trichloroacetic acid, 10% w/v. (2) Trichloroacetic acid, 5% w/v. (3) 80% ethanol. (4) Ethanol-chloroform, 3:1. (5) Silica-free N NaOH or N KOH. (6) 2.5N HCl. (7) Trichloroacetic acid, 30% w/v. (8) Reagents as in Method I, *a*) 3-10 (p. 226) for phosphorus estimation. (9) Perchloric acid, 6% v/v. (10) 6% w/v orcinol (recrystallized from benzene) in 96% alcohol. (11) 0.02% $FeCl_3 \cdot 6H_2O$ in conc. HCl. (12) Freshly prepared orcinol reagent (Kerr and Seraidarian's (16) modification of Mejbaum's (19) reagent): 1 ml of solution (10) added to 10 ml of solution (11). (13) Dische's (13) diphenylamine reagent: 1% w/v diphenylamine (purified (*a*) by dissolving in benzene and passing through a column of alumina (14); (*b*) by redistillation (20), or (*c*) by twice recrystallization from ethanol or methanol) in purified glacial acetic acid containing 2% v/v conc. H_2SO_4.

b) Tissue culture method.—The amount of tissue required is limited by the quantity of DNA which can be determined by either phosphorus or desoxyribose analyses. DNAP can be determined ((*e*), below) in amounts of tissue containing not less than 10 μg DNA; for the desoxypentose estimation at least 5 times this amount is desirable, and it may therefore be necessary to pool the contents of 2 or more roller tubes.

c) Extraction of acid-soluble and lipid phosphorus.—The fluid medium is discarded and the cultures are briefly washed in the roller tube with physiologic saline solution. The tissue and coagulum are detached from the glass and washed with 2 ml of ice-cold 10% trichloroacetic acid into a Pyrex tube and centrifuged. The roller tube is washed out and the tissue re-extracted with 2 more 2 ml portions of trichloroacetic acid. The tissue residue is washed with 80% and with absolute ethanol, twice extracted at 70 C with ethanol-chloroform and washed with ether.

d) Separation of pentose and desoxypentose nucleic acid fractions.—The dry tissue residue is incubated at 37 C for at least 15 hr with 1 ml of N NaOH. Unhydrolyzed DNA is precipitated by adding 1.5 ml of water, 0.5 ml of 2.5N HCl and 0.6 ml of 30% trichloroacetic acid and centrifuging. This precipitate (*P1*) is washed twice with 0.5 ml portions of 5% trichloroacetic acid. The combined supernatant and washing (*S1*) contain the hydrolyzed RNA.

e) Phosphorus estimations.—These are carried out on the precipitate *P1* for determination of DNAP, and on the supernatant *S1* for RNAP. For ashing the supernatant, 0.3 ml of 60% $HClO_4$ is required.

f) Pentose and desoxypentose estimations.—(1) Samples of the supernatant *S1* may be used for determination of pentose by a modification of the method of Mejbaum (19). For this, 2 ml of *S1* is treated with 2 ml of orcinol reagent and the mixture heated for 40 min at 100 C in a boiling water bath. The green color is stable and the intensity may be read in a colorimeter or spectrophotometer.

(2) The precipitate *P1* is extracted with 1 ml of 5% trichloroacetic acid for 15 min at 90 C (25, 26) or with 1 ml of 6% perchloric acid under the same conditions (27). The longer procedure, with more concentrated acid (2 extractions at 80 C for 30 min, using 10% perchloric acid), recommended by Ogur and Rosen (21) for animal tissues seems to have no advantage over the simpler method. Only 80-85% of the total P of the precipitate *P1* is extracted by trichloroacetic acid, while 100% is extracted by perchloric acid under Schneider, Hogeboom and Ross's conditions (18, 30). Perchloric acid was originally recommended as an alternative extractant to trichloroacetic acid because trichloroacetic acid has a high absorption in the ultraviolet below 270 mμ and so is objectionable as a component of nucleic acid extracts intended for ultraviolet absorption measurements (21). Schneider, Hogeboom and Ross (27) use perchloric acid extracts for determination of DNA by the diphenylamine procedure. In contrast to the findings with P, the amount of diphenylamine-reacting material extracted by perchloric acid is not greater than with trichloroacetic acid (30). Standard DNA solutions for calibrating these methods should be prepared by dissolving the nucleic acid in 5% trichloroacetic acid or 6% perchloric acid, respectively, with heating at 90 C for 15 min. The light absorption in the Dische test is about 10% greater with these solutions than with DNA dissolved in water with the aid of alkali (23).

The cooled trichloroacetic acid or perchloric acid extract is made up to exactly 1 ml, 4 ml of diphenylamine reagent is added and the mixture heated for 6 min at 100 C, cooled rapidly and the color read in an absorptiometer or spectrophotometer. The intensity of the color, and consequently the sensitivity of the method, is greatly increased by incubating the reaction mixture in stoppered tubes at 37 C for 16 hr instead of heating at 100 C for 6 min (22).

B. METHOD OF HULL AND KIRK (15)

A modification of the Schmidt and Thannhauser (24) technique is used for extraction of the nucleic acids.

a) *Reagents.*—(1) As in method II, *a*), 1–8 (p. 227). (2) HNO_3, conc.

b) *Tissue culture methods.*—A whole chick embryo heart, 4–10 mg, is cut into fragments and implanted in each roller tube in a minimum amount (0.1 ml) of dilute (1:9) plasma. As fluid medium, 4 ml of serum-embryo extract is used. The cultures for analysis are washed 3 times with 1 ml of isotonic saline.

c) *Separation of nucleic acid fractions.*—The roller tube is cooled and 1 ml of cold 10% trichloroacetic acid added. After 10 min extraction the supernatant is filtered through a micro Buchner funnel and the extraction repeated once. The tissue is washed 3 times with 5% trichloroacetic acid and phospholipids are removed by extraction with alcohol (26), substituting filtration for centrifugation. The filter paper and precipitate are transferred back to the culture tube, 1 ml of 1N NaOH is added and the tube incubated at 37 C for 18 hr. The soluble materials are washed into a centrifuge tube, cooled and neutralized with cold 6N HCl. To this solution is added $^1/_6$ vol of cold 30% trichloroacetic acid and the precipitate of DNA allowed to flocculate for 1 hr in a refrigerator. The precipitate is centrifuged down and analysed for DNAP. The supernatant fluid is filtered and used for RNAP analysis.

d) *Phosphorus determination.*—The sample is digested in a micro Kjeldahl flask with 1 ml of conc. HNO_3 for $2^1/_2$ hr, removed and cooled. Two drops of 60% $HClO_4$ is added and digestion continued for 20 min over a low flame. By increasing the heat, the solution is evaporated almost to dryness, cooled and diluted with 0.3–0.4N H_2SO_4 to a volume containing 2–5 μg of P/ml. These samples are washed into a micro separator (17) with 2 ml of 0.2N H_2SO_4, analysed according to Berenblum and Chain (1) and light absorption measured in a Beckman spectrophotometer at 720 mμ.

C. METHOD OF STEELE, SFORTUNATO AND OTTOLENGHI (28)

Samples containing 8.6–58.0 μg of RNA and 1.6–15.1 μg of DNA/-mg wet weight can be analysed by this method.

a) *Reagents.*—(1) Trichloroacetic acid, 10%. (2) Trichloroacetic acid, 5%. (3) Dische's diphenylamine reagent (p. 227). (4) Mejbaum's (19) orcinol reagent: 100 μg of orcinol dissolved in 10 ml of conc. HCl containing 0.1% $FeCl_3 \cdot 6H_2O$.

b) *Procedure.*—Special micro equipment is used (Fig. 1 in (28)). Tissue sample is homogenized for 1 min with 0.03 ml of cold distilled water in a micro Potter-Elvehjem homogenizer, using a stirrer revolving at 1500 rpm. The pestle is moved a short way up the tube and another 0.03 ml of water added and sucked below the pestle. Two 0.08 ml portions of cold 10% trichloroacetic acid are added similarly and the washed pestle is removed from the tube. A stirring thorn is used to mix the contents of the micro tubes at each subsequent washing. The micro tubes are centrifuged at 0.5 C for 15 min in a Sorvall Model G angle head centrifuge.

The trichloroacetic-acid precipitated material is washed twice in the cold with 0.10 ml portions of 10% trichloroacetic acid and once with 0.10 ml of 5% trichloroacetic acid.

The nucleic acids are extracted by heating the sediment with 0.05 ml of 5% trichloroacetic acid, after the method of Schneider (25). An additional 0.05 ml is used for washing. The combined extracts are collected in a calibrated extract pipet and made up to 0.100 ml.

A 0.033 ml aliquot is taken for the Dische diphenylamine reaction. Reagent is drawn in to the 0.100 ml mark, the special pipet capped and heated in a boiling water bath. The colored solution is read in the Beckman spectrophotometer, using microcuvets of 1 cm light path.

A 0.033 ml aliquot for RNA determination is emptied into 0.22 ml of 5% trichloroacetic acid in a 13×100 mm tube. To this is added 0.25 ml of Mejbaum's orcinol reagent and the color developed and read in a microcuvet. Correction for color contributed by DNA is applied by subtracting 0.023 μg of ribose for each 1.0 μg of DNA in the aliquot.

D. METHOD OF DACKERMANN AND PATTERSON (3)

This method for micro determination of nucleic acids in small amounts of tissue employs only ordinary microchemical apparatus and covers the range 10–20 μg PNA and 1–2 μg DNA in tissue samples containing 18–26 μg of N.

After homogenization in cold 95% ethanol, the tissue is extracted successively in several portions of warm 95% ethanol and ether, cold (0°) 10% trichloroacetic acid and hot (90°) 5% trichloroacetic acid (fraction *A*). Phosphorus determinations (range 0.1–1.0 μg P) are made on aliquots of fraction *A* and on the tissue residue. Orcinol (range 0.3–1.8 μg PNA determination) and diphenylamine (range 0.3–1.3 μg DNA determination) tests are also carried out.

REFERENCES

1. Berenblum, I., and Chain, E.: An improved method for the colorimetric determination of phosphate, Biochem. J. 32: 295, 1938.
2. Berenblum, I.; Chain, E., and Heatley, N. G.: The study of the metabolic activities of small amounts of surviving tissue, Biochem. J. 33: 68, 1939.
3. Dackermann, M. E., and Patterson, E. K.: Abst., Am. Chem. Soc., 116th Meeting, Div. Biol. Chem., 36C–37C, 1949.
4. Davidson, J. N.: Some factors influencing the nucleic acid content of cells and tissues, Cold Spring Harbor Symposia 12: 50, 1947.
5. Davidson, J. N., and Leslie, I.: A new approach in the biochemistry of growth and development, Nature, London 165: 49, 1950.
6. Davidson, J. N., and Leslie, I.: Nucleic acids in relation to tissue growth: A review, Cancer Research 10: 587, 1950.
7. Davidson, J. N., and Waymouth, C.: Factors influencing the nucleoprotein content of fibroblasts growing in vitro, Biochem. J. 37: 271, 1943.
8. Davidson, J. N., and Waymouth, C.: Factors in pancreatin which influence the nucleoprotein content of fibroblasts growing in vitro, Quart. J. Exper. Physiol. 33: 19, 1944.
9. Davidson, J. N., and Waymouth, C.: Nucleic acids and tissue growth, Nutrition Abst. & Rev. 14: 1, 1944.
10. Davidson, J. N., and Waymouth, C.: The nucleoprotein content of fibroblasts growing in vitro: 2. The effects of tissue extracts, Biochem. J. 39: 188, 1945.
11. Davidson, J. N., and Waymouth, C.: The nucleoprotein content of fibroblasts growing in vitro: 3. The use of defatted media, Biochem. J. 40: 568, 1946.
12. Davidson, J. N.; Leslie, I., and Waymouth, C.: The nucleoprotein content of fibroblasts growing in vitro: 4. Changes in the ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) content, Biochem. J. 44: 5, 1949.
13. Dische, Z.: Über einige neue charakteristische Farbreaktionen der Thymonukleinsäure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen, Mikrochemie 8: 4, 1930.
14. Harris, R. J. C.: Personal communication, 1950.
15. Hull, W., and Kirk, P. L.: Tissue culture studies: II. The relationship of nucleic acid increase to the growth of cells in vitro, J. Gen. Physiol. 33: 327, 1950.
16. Kerr, S. E., and Seraidarian, K.: The separation of purine ribosides from free purines and the determination of the purines and ribose in these fractions, J. Biol. Chem. 159: 211, 1945.
17. Kirk, P. L., and Danielson, M.: A liquid-liquid microextractor for solvents lighter than water: Use in phosphate analysis, Indust. & Engin. Chem. (Anal. Ed.) 20: 1122, 1948.
18. Leslie, I.: Personal communication, 1950.
19. Mejbaum, W.: Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure, Ztschr. f. physiol. Chem. 258: 117, 1939.
20. Nowinski, W. W.: Personal communication, 1950.
21. Ogur, M., and Rosen, G.: The nucleic acids of plant tissues: I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid, Arch. Biochem. 25: 262, 1950.
22. Patterson, E. K.: Personal communication, 1950.

23. Petermann, M. L.; Alfin-Slater, R. B., and Larack, A. M.: The nucleic acid distribution in normal and leukemic mouse spleen, Cancer 2: 510, 1949.
24. Schmidt, G., and Thannhauser, S. J.: A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phospho-proteins in animal tissues, J. Biol. Chem. 161: 83, 1945.
25. Schneider, W. C.: Phosphorus compounds in animal tissues: I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid, J. Biol. Chem. 161: 293, 1945.
26. Schneider, W. C.: Phosphorus compounds in animal tissues: III. A comparison of methods for the estimation of nucleic acids, J. Biol. Chem. 164: 747, 1946.
27. Schneider, W. C.; Hogeboom, G. H., and Ross, H. E.: Intracellular distribution of enzymes: VII. The distribution of nucleic acids and adenosinetriphosphatase in normal mouse liver and mouse hepatoma, J. Nat. Cancer Inst. 10: 977, 1950.
28. Steele, R.; Sfortunato, T., and Ottolenghi, L.: A micromethod for the determination of the nucleic acids, J. Biol. Chem. 177: 231, 1949.
29. Waymouth, C.: Methods of estimating the growth of tissue cultures, Exper. Cell Research, Supp. 1, p. 460, 1949.
30. Waymouth, C., and Laurence, G. D.: Unpublished observations, 1950.
31. Willmer, E. N.: Carbohydrate metabolism of chick fibroblasts in vitro, J. Exper. Biol. 18: 237, 1942.

METHODS FOR STUDY OF ORGANIZED GROWTH IN VITRO

Introduction

HONOR B. FELL, *Strangeways Research Laboratory, Cambridge, England*

It is a familiar fact that when a fragment of tissue is cultivated in vitro in a suitable nutritive medium, amoeboid cells wander into the culture medium to form a zone of undifferentiated tissue around the parent fragment. By transplantation and subdivision this unorganised growth can sometimes be maintained for years. It has been the subject of a vast literature, and many biologists are hardly aware that a second type of growth may also take place in tissue culture which has been termed organised growth.

In organised growth the explanted tissue retains its characteristic histological structure and grows as if it were still part of the animal from which it was taken; if obtained from an early embryo it usually continues its histological and sometimes also its anatomical development (7, 10) while in culture.

Organised growth was first observed 36 years ago by Thomson (19), and since then many types of undifferentiated tissue have been cultivated, e.g., cartilage (4, 17), bone (6), teeth (12), eye (3, 18), ear (5), metanephros (15), salivary gland (1), hair (13, 16), gonads (14; and see p. 238) and endocrine glands (2; and see pp. 237 and 241). Publications on this subject have been reviewed elsewhere (8). So far, cultures of organised tissue have been used almost entirely for morphological studies, and nearly all physiological investigations on growth in vitro refer to unorganised cultures. Similar research on the physiology of differentiated tissue in vitro might be very rewarding and provide information about the individual metabolism of the various tissues and organs of the body which could not be obtained in any other way. In particular, cultures of organised tissues are well adapted to nutritional studies and are known to be very sensitive to alterations in the composition of the medium (9, 11). It is to be hoped that more work of this type will be undertaken in the future.

REFERENCES

1. Borghese, E.: The development in vitro of the submandibular and sublingual glands of Mus musculus, J. Anat. 84: 287, 1950.

2. Carpenter, E.: Differentiation of chick embryo thyroids in tissue culture, J. Exper. Zool. 89: 407, 1942.
3. Dorris, F.: Differentiation of the chick eye in vitro, J. Exper. Zool. 78: 385, 1938.
4. Fell, H. B.: Experiments on the differentiation in vitro of cartilage and bone: Part I, Arch. f. exper. Zellforsch. 7: 390, 1928.
5. Fell, H. B.: The development in vitro of the isolated otocyst of the embryonic fowl, Arch. f. exper. Zellforsch. 7: 69, 1928.
6. Fell, H. B.: The osteogenic capacity in vitro of periosteum and endosteum isolated from the limb skeleton of fowl embryos and young chicks, J. Anat. 66: 157, 1932.
7. Fell, H. B.: The origin and developmental mechanics of the avian sternum, Phil. Tr. Roy. Soc., s.B. 229: 407, 1939.
8. Fell, H. B.: Histogenesis in Tissue Culture, in Bourne, G. H. (ed.): *Cytology and Cell Physiology* (Oxford: Clarendon Press, 1950).
9. Fell, H. B., and Mellanby, E.: Effects of hypervitaminosis A on foetal mouse bones cultivated in vitro, Brit. M. J. 2: 535, 1950.
10. Fell, H. B., and Robison, R.: The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro, Biochem. J. 23: 767, 1929.
11. Gaillard, P. J.: Hormones Regulating Growth and Differentiation in Embryonic Explants, in Actualités scientifiques et industrielles (Paris: Hermann et Cie, 1942).
12. Glasstone, S.: A comparative study of the development in vivo and in vitro of rat and rabbit molars, Proc. Roy. Soc., London, s.B. 126: 315, 1938.
13. Hardy, M. H.: The development of mouse hair in vitro with some observations on pigmentation, J. Anat. 83: 364, 1949.
14. Martinovitch, P. N.: The development in vitro of the mammalian gonad: Ovary and ovogenesis, Proc. Roy. Soc., London, s.B. 125: 232, 1938.
15. Rienhoff, W.: Development and growth of the metanephros or permanent kidney in chick embryos, Bull. Johns Hopkins Hosp. 33: 392, 1922.
16. Strangeways, D. H.: The growth of hair in vitro, Arch. f. exper. Zellforsch. 11: 344, 1931.
17. Strangeways, T. S. P., and Fell, H. B.: Experimental studies on the differentiation of embryonic tissues growing in vivo and in vitro: I. The development of the undifferentiated limb-bud (*a*) when subcutaneously grafted into the post-embryonic chick, and (*b*) when cultivated in vitro, Proc. Roy. Soc., London, s.B. 99: 340, 1926.
18. Strangeways, T. S. P., and Fell, H. B.: Experimental studies on the differentiation of embryonic tissues growing in vivo and in vitro: II. The development of the isolated early embryonic eye of the fowl when cultivated in vitro, Proc. Roy. Soc., London, s.B. 100: 273, 1926.
19. Thomson, D.: Some further researches on the cultivation of tissues in vitro, Proc. Roy. Soc. Med., Vol. 7, Marcus Beck Lab. Rep., p. 21, 1914.

Techniques of Bone Cultivation

HONOR B. FELL, *Strangeways Research Laboratory, Cambridge, England*

The technique of cultivating organised tissues is simple. The main difficulty usually lies not in maintaining the cultures but in preparing the tissue for explantation; this part of the work often involves the dissection, without damage, of rudiments from young embryos and requires practice and some manual dexterity. The methods used at the Strangeways Research Laboratory for cultivating differentiated bone and cartilage follow.

WATCH GLASS METHOD

This is a modification (4) of a technique devised by the late Dr. T. S. P. Strangeways and has been successfully used for many different types of organised cultures besides bone and cartilage. The work can be safely done on an open bench in an ordinary clean room and it is unnecessary to wear a cap or mask. With careful manipulation the operator is rarely troubled by bacterial contamination, but it is realised that in a hot dusty atmosphere more stringent precautions may be necessary.

To prepare the culture vessels, a circle of absorbent cotton wool is cut to fit a Petri dish, and a round hole about 1 in. in diameter is removed from the centre of the cotton wool to allow transillumination of the culture. A watch glass is placed in the Petri dish over this central hole and the dish is sterilised by dry heat. A convenient size for the Petri dish is 8 cm diameter of top dish × 10 mm deep, with watch glasses 4 cm in diameter.

Sterile distilled water (10 cc) is pipetted into each dish and is absorbed by the cotton wool, a very efficient moist chamber being thus obtained. A useful culture medium for many tissues, both avian and mammalian, is a mixture of equal parts of plasma and a concentrated saline extract of 12–14 day chick embryo. The plasma should not be used after more than a week's storage in the ice box, and we prefer to make fresh embryo extract on each culture day. Four drops of plasma are first deposited in all the watch glasses with a capillary pipet. The same amount of embryo extract is then added to not more than 2 vessels at a time, and the medium is thoroughly mixed and spread with a fine glass rod. The culture vessels are now ready for use.

The watch glass method is intended for relatively large explants and is unsuitable for very small fragments which grow and develop better in an ordinary hanging drop preparation. There is often an optimal stage of development at which a rudiment should be explanted and which must be determined empirically for each tissue. If it is desired to work with a highly differentiated tissue, it is sometimes best to explant the rudiment at an earlier period of development and allow it to differentiate in vitro to the stage required. Of skeletal tissues, we have obtained successful results with the blastema of the limb bud from 4 day chick embryos (2), with isolated bone rudiments from slightly older chick embryos

(4, 5), with the embryonic budgerigar sternum (1) and recently with the long bones of late foetal mice (3).

The tissue is prepared on a dissecting binocular microscope, to the stage of which a small, detachable glass shade is fitted to prevent dust particles from falling on the tissue during dissection. The dissection itself is done with a sewing needle mounted in a thin glass rod and a very fine cataract knife, ground to a sharp, flexible point by the investigator himself. While sharpening the knife, it is advisable to examine it under the dissecting binocular at short intervals; unless the tip of the blade is thin and flexible it will snap easily and it will also inflict considerable damage on the delicate explants. When the rudiment has been freed from the surrounding tissue, it is washed several times in Tyrode solution, but it should not be left soaking in saline for longer than is absolutely necessary.

The explants are sucked up in a medium-sized capillary pipet and deposited on the surface of the clot in a small drop of saline; it is unwise to grow more than 4 explants in 1 dish, as this prolongs the manipulation and increases the risk of infection. The Petri dish is then put on the stage of the dissecting binocular beneath the glass shade, the lid is removed and the surplus saline sucked away from the tissue with a very fine capillary pipet made especially for this purpose; care should be taken to avoid damaging the clot with the fine pipet. The Petri dish is then put in the incubator. The dishes should not be piled on each other, as this produces a heavy condensation of water droplets on the inner surface of the lids. In some incubators this condensation may appear even if the dishes are not piled; this may be overcome by covering the shelves of the incubator with $^1/_2$ in. board.

Every 2–3 days the explants are transferred to freshly prepared culture vessels. The clot is pulled gently away from the rudiment so that the zone of outgrowth is ruptured, and the tissue can then be detached from the clot with the knife and needle; it is important not to leave any old clot attached to the explant. The rudiments are sucked into a medium-sized capillary pipet, quickly rinsed in a drop of saline and transferred to fresh medium.

HANGING DROP METHOD

To prepare large numbers of smaller ossifying cultures, ordinary large hanging drop preparations ($3 \times 1^1/_2$ in. hollow ground slides and $1^1/_4$ in. square cover slips) are useful. Osteogenic tissue may be obtained in the following way. The tibia of a 13 day fowl embryo is cleared of muscle, and the 2 cartilaginous ends are cut off and rejected. The tube of diaphyseal bone is split lengthwise, the marrow removed and the bone cut into rectangular explants. Each explant is grown for 2–3 days, when it will have become surrounded by a broad zone of outgrowth; the original bone is then carefully dissected out, the ring of outgrowth cut from the clot in the usual way and transplanted to a medium composed of 2 drops of fowl plasma and 1 drop of concentrated extract of 12–14 day chick embryo. The central hole left by removal of the original bone soon heals and new bone and cartilage differentiate in the interior of the tissue; the culture is transferred to fresh medium every 2–3 days by the ordinary method of transplantation

REFERENCES

1. Fell, H. B.: The origin and developmental mechanics of the avian sternum, Phil. Tr. Roy. Soc., s.B. 229: 407, 1939.
2. Fell, H. B., and Canti, R. G.: Experiments on the development in vitro of the avian knee-joint, Proc. Roy. Soc., London, s.B. 116: 316, 1934.
3. Fell, H. B., and Mellanby, E.: Effect of hypervitaminosis A on foetal mouse bones, Brit. M. J. 2: 535, 1950.
4. Fell, H. B., and Robison, R.: The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro, Biochem. J. 23: 767, 1929.
5. Fell, H. B., and Robison, R.: The development and phosphatase activity in vivo and in vitro of the mandibular skeletal tissue of the embryonic fowl, Biochem. J. 24: 1905, 1930.

Culture of Infantile Endocrine Glands of Rats by Watch Glass Technique in a Moist Chamber

PETAR N. MARTINOVITCH, *Serbian Academy of Science*

ADRENALS

This technique applies to infantile rats up to 5 days of age. The organs can be maintained in vitro for at least 60 days. Adrenals from older rats, up to 15 days after birth, have also been cultured, but no satisfactory results have been obtained. Adrenals of 6, 7 and 8 day old rats "grew" better than the same glands of 12–15 day old animals but they did not thrive as well as those taken from 5 day old rats. The reason for this marked difference in adaptability to the conditions in vitro of adrenals coming from animals of slightly different ages remains unexplained.

PROCEDURE

Whole adrenals of 5 day old rats are cultured by Fell's watch glass technique (p. 235) in heparinized rat plasma, chicken plasma and concentrated chick embryo extract in proportions of 6 to 2 to 3 drops at 34 C. To this medium 4 drops of Gey's balanced saline solution is added for the purpose of lowering the protein concentration (Gey, in a personal communication). The explant is mounted on the surface of the plasma clot. As the rat plasma easily undergoes liquefaction, the explant soon sinks to the bottom of the watch glass. To avoid this, the explant is transferred to a different position on the plasma clot at 24 hr intervals. After every 7 days of cultivation, all the explants are placed on the surface of a fresh nutritive medium.

Comment.—Explanted adrenals show visible signs of activity as manifested by constant migration of cells from the mother explant into the surrounding medium. The rate of migration is somewhat slowed by the saline solution which makes the coagulum less firm (1); and, of course, an incubating temperature of 34 C also reduces the activity of the peripheral cells of the explant, as does the low concentration of the embryo-juice present in the medium. Attempts at mounting the explants on the tunica albuginea of the testis (see discussion of the anterior pituitary, below) failed, and this modification of the technique had to be abandoned.

After 2 months of cultivation a large number of cortical cells survive, and they appear to be quite healthy. All explants proved to be transplantable after 6 weeks of in vitro life. Older explants have not been transplanted, but with them, too, successful takes may be possible. The medullary portion of the organ, so far as we have been able to tell, survives only a relatively short time.

GONADS

By the use of the watch glass technique infantile rat and mouse ovaries and testes can be kept in vitro for several months, probably for an indefinite period (2, 3). In the rat ovary, egg cells of the type of *residual ova* were still evident when cultures were discontinued after 107 days of incubation.

ANTERIOR PITUITARY

Whole or halved anterior pituitaries of infantile rats can be cultivated in vitro by the watch glass technique for at least 3 months.

PROCEDURE

The medium is heparinized rat plasma, chicken plasma and concentrated chicken embryo extract in the proportion of 6 to 2 to 3 drops at 34 C. Instead of being placed directly on the plasma clot, the explant is mounted on a piece of the tunica albuginea of the testis taken from animals 16–18 days old. A rectangular piece of albuginea, about 2×3 mm in area, preferably with a few seminiferous tubules adhering to it, is spread on the surface of the plasma clot with the epithelial layer facing upward and the pituitary is placed on the top of it (Fig. 1). In the course of time the 2 pieces of tissue fuse, but they are easily distinguished by the way they break transmitted light. When the albuginea grows old, usually after a month of in vitro life, the 2 pieces of tissue are separated by several gentle clean cuts along the line of fusion with a pair of fine iridectomy scissors, with care to avoid crushing of the cells. The pituitary is then mounted again on a fresh piece of albuginea.

The albuginea sinks fairly easily in the plasma clot and a pool of liquid medium surrounding the explant is quickly formed. This assures adequate nutrition for the pituitary. Care, however, must be taken that the explant does not sink to the bottom of the watch glass (see procedure for the adrenal gland, above) or, on the other hand, that the pool of the liquid medium surrounding the explant is so small that it cannot reach the pituitary. Once a week the explant is transferred to freshly prepared nutritive medium.

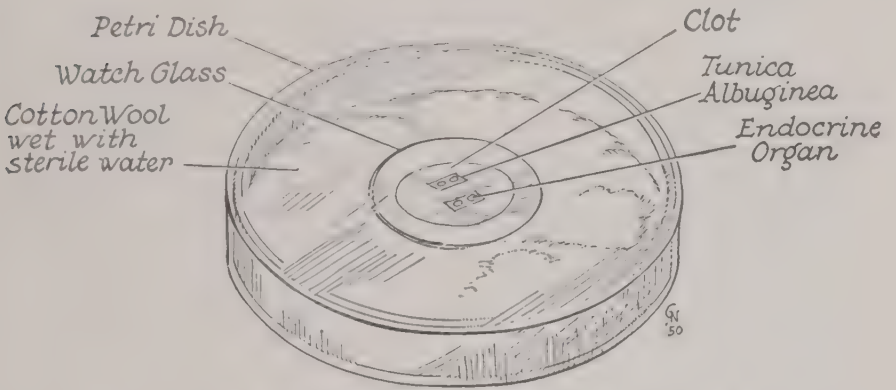

FIG. 1.—Watch glass culture using Petri dish as a moist chamber.

Comment.—Pituitaries cultivated for 6 weeks were found to be transplantable in vivo. Young rats hypophysectomized for 3–11 months, whose growth rates had become definitely plateaued as a result of the operation, were found to resume a marked increase in growth immediately following the implantation of pituitaries cultured for 32–35 days in vitro (4). Explants cultivated for 6 weeks appeared healthy and a large number of acidophilic cells, full of fuchsin-staining granules, and chromophobes were present. In fact, preliminary observations have revealed healthy-looking acidophilic cells full of granules in explants after 3 months of culture in vitro. However, Sevringhaus' stain, applied after 4 weeks of cultivation, failed to reveal basophil cells in explanted pituitaries.

Fragments of anterior pituitaries of adult rats can also be cultured with success by the same technique.

The advantages of growing rat pituitaries in vitro with the use of a piece of albuginea as an additional substrate appear to be that: (1) the explant remains healthy for a longer period; (2) albuginea, in a way not yet understood, helps the explanted gland preserve its original pattern; (3) so far as we are aware, migration of cells from the pituitary explant on or into the tissues of the albuginea does not take place.

PINEAL BODY

In a limited series of experiments, epiphyses cerebri from 15 day old rats were successfully cultured in vitro for at least 3 weeks by the watch glass technique.

REFERENCES

1. Gaillard, P. J.: Growth, differentiation and function of explants of some endocrine glands, Symp., Soc. f. exper. Biol. 2: 139–145, 1948.
2. Martinovitch, P. N.: The development in vitro of the mammalian gonad, ovary and ovogenesis, Proc. Roy. Soc., London, s.B. 125: 232–249, 1938.
3. Martinovitch, P. N.: The effect of subnormal temperature on the differentiation and survival of cultivated in vitro embryonic and infantile rat and mouse ovaries, Proc. Roy. Soc., London, s.B. 128: 138–143, 1939.
4. Martinovitch, P. N.: Anterior pituitary explants of infantile rats grafted in the anterior eye chamber of hypophysectomized hosts, Nature, London 165: 33–35, 1950.

Technique of Grafting Tissues and Organs, Including Those Cultivated in Vitro, into Anterior Eye Chamber of Rats and Mice

PETAR N. MARTINOVITCH, *Serbian Academy of Science*

The technique of introducing infantile organs, especially the anterior pituitary, in the anterior eye chamber of small laboratory animals with a glass pipet or a trocar under the dissecting microscope is difficult owing to the size of the eye and the extreme softness of the organ. The following method gives excellent results.

PROCEDURE

The cornea is pierced with a sharp, hard steel lancet about halfway between pupil and sclera, but somewhat closer to the sclera. This is necessary to prevent iris prolapse which may result from internal ocular pressure. The position of the corneal puncture is easily ascertained if the extruded aqueous humor is removed with a piece of slightly moistened cotton wool. The corneal opening is enlarged to the desired size by inserting 1 blade of a pair of iridectomy scissors, of the type used for experimental work in embryology but adapted for the eye, through the puncture made with the lancet Cuts with the scissors through the cornea should be made without injuring the iris. To make certain of the size of the corneal opening, a fine glass rod is introduced and moved back and forth lengthwise to the cut. A crescent-like opening is better than a straight one. An opening that is too large should be avoided because this delays healing of the wound. Once the size of the opening is ascertained, the piece of tissue to be grafted is brought to the lower lip, i.e., on the scleral side of the opening, preferably with a very small, thin and flexible platinum spatula. With 1 stroke of a dental pulp remover the piece of tissue is pushed into the anterior eye chamber. The sharp teeth of the pulp remover should be blunted but not entirely removed by pressing them gently on a fine-grain grindstone, otherwise the same teeth that help push the tissues into the eye may pull them out on withdrawal of the instrument. Once the tissue is securely lodged in the interior of the chamber, it can be moved to the opposite corneoscleral angle by gentle pressure on the surface of the cornea with a glass rod or blunt surgical needle. As many as 8 pieces of tissue of transplantable size can be introduced in the same eye without permanently injuring the organ.

Explants of endocrine organs, especially after prolonged cultivation, toughen in vitro, and they are more successfully introduced into the eye if they are pushed with a sharp-pointed steel needle.

Organ Culture Technique Using Embryologic Watch Glasses

PETER J. GAILLARD, *State University of Leyden*

HUMAN PARATHYROID GLAND

Removal of glands.—Within 3 hr after death, *thyroid* glands are removed aseptically from full term newborn babies and immediately placed and continually kept in 10% placental vein serum in Gey's balanced saline solution cooled to 4 C. On a sterile glass plate cooled to 4 C the adhering tissues at the dorsal side and at the edges of the gland are removed under the dissecting microscope. Embedded in loose connective or fatty tissue a number of small organs approximately 1×1 mm may be found, such as extra thyroid glands, lymph nodes, thymus glands, as well as true parathyroid glands. Neither topographic relations nor crude morphologic criteria are sufficient to decide whether the organs are parathyroid glands or not; consequently all organs have to be cultured until histologic control sections or their behavior in vitro make identification absolutely conclusive. The glands are carefully freed from excess connective tissue and each is placed in a separated drop of the serum-saline mixture on another sterile, cooled glass plate and cut into 4–8 fragments about 0.25–0.50 mm³.

CULTURE TECHNIQUE

According to previous work (2, 3), the organotypic structure of many tissues can best be preserved by cultivating the fragments on the top of a very *weak* clot composed of *homologous* and aseptically prepared "fluids" obtained from individuals of the same or of older but *not* of younger age as that from which the tissue is prepared. In our experience, the most suitable medium for parathyroid gland tissue is: 5–15% adult human blood plasma containing 0.5 cc of a 0.1% solution of heparin/10 cc of blood; 10% placental vein serum; 75–65% Gey's balanced saline solution and 10% human fetal brain press juice.

To prepare the press juice, brain tissue of a newborn fetus is removed aseptically as soon as possible after death. It is cut into pieces of approximately 1 cm³ and placed in a Petri dish at 4 C for 24 hr to facilitate separation of tissue fluid. The tissue is then minced with a tissue press, an equal amount of Gey's saline solution is added and the mixture centrifuged for 15 min at 6000–8000 rpm. The press juice is pipetted into Pyrex glass tubes and stored at −20 C.

The components are mixed in the order given immediately before transfer of 0.75 ml of the medium to each embryologic watch glass (Fig. 1, *1*) to be used in the experiment. Immediately after onset of coagulation the fragments of each organ being used are placed on the top of the clot in a watch glass, except 1 fragment of each organ which is reserved for histologic study. The dishes are sealed with a glass plate, using paraffin, and placed in an incubator at 37 C. Three days later the explants are transferred to fresh watch glasses, or the fragments can be planted separately on the top of minute drops of the same kind of medium on mica cover slips and grown as hanging drop cultures. As a rule, the cultures must be replenished twice a week.

Behavior of the explants.—Parathyroid tissue appears to be very sensitive to the damage caused by cutting of the fragments. Consequently, during the first day of explantation the peripheral cell layers will disintegrate completely, but at the same time, in the center of the fragments, a well bordered "ball" of translucent and healthy tissue may be seen. In the meantime, the clot will become completely liquefied. At the end of the third day of cultivation the fragments can be sucked into a sterile pipet, washed in the serum-saline mixture at room temperature to remove the débris of the disintegrated tissue, and then these fully epithelialized explants can be transferred onto a fresh medium.

During the cultivation procedure, most of the explants gradually increase in volume by the apposition of newly formed epithelial cell layers, and as long as the typical parathyroid structure remains intact the medium always will become completely liquefied. Generally, organotypic structure can be preserved for 30–60 days.

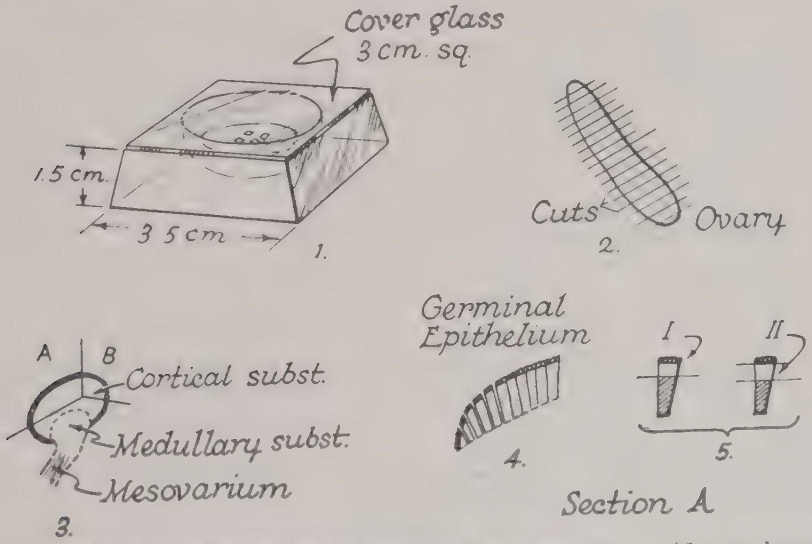

FIG. 1.—Embryologic watch glass method for organ culture. (See explanation in text.)

Throughout the procedure, great care must be used to avoid injury to the enclosing epithelial membrane, which is essential to successful organized growth.

Use in human replacement therapy.—With P. J. Kooreman, successful attempts have been made, especially in younger individuals, to graft the cultivated parathyroid fragments in patients suffering from postoperative tetany (3, 4, 9). In cultivation of the tissue for these purposes, the blood plasma of the medium, at the first or second transfer, was replaced by blood plasma from the prospective recipient. At the next transfer the placental vein serum was replaced by the blood serum from the future host, and finally the fetal brain press juice was completely omitted. Following this the fragments were cultivated for at least another 10 days in the medium derived from the recipient.

The grafting of parathyroid "organs" adapted to the prospective host was done by pipetting fragments, washed and suspended in 10% recipient serum in Gey's saline solution, into the lumen of the perivascular lymph space associated with 1 of the axillary veins, as described by Stone, Owings and Gey (11).

The procedure for adapting tissues to be grafted was chosen on the basis of observations on the use of homologous vs. heterologous media in tissue culture. For instance, mammalian tissues show a poor initial outgrowth when cultivated exclusively in avian media but after some time seem to become "adapted" to the foreign proteins and can then be grown very satisfactorily. Moreover, in the transplantation work done by Gassul (8) between frogs, by Erdmann (1) between frogs and toads and later by Stone, Owings and Gey (11) in dogs and also in man, the idea of adapting tissues to the recipient by adding the serum of the prospective host to the culture medium was worked out in greater detail. However, these methods were not quite adequate to keep the organotypic structure intact. A comparative study of the changes in the tissue proteins resulting from cultivating tissues in media with proteins from various animals was made by Langman (10).

HUMAN FETAL OVARY

Removal of the organs.—Ovaries are removed aseptically from 4-9 month old human fetuses within 6 hr after death. The abdominal wall is opened by a transverse incision, with careful avoidance of any bleeding into the abdominal cavity. The fetus is placed in the Trendelenburg position to facilitate removal of the ovaries, adhering fallopian tubes and part of the mesovarium. These are transferred to 100 ml of a sterile mixture of 10% placental vein serum in Gey's balanced saline solution. Immediately, the organs are placed in separated drops of the serum-saline mixture on a sterile glass plate cooled to 4 C. Under the dissecting microscope the tubes and most of the mesovarium are removed. The organs are then cut into slices approximately 1 mm thick perpendicular to the long axis of the organ (Fig. 1, *2*). The cortical parts of these slices are divided (*3*) and pieces *A* and *B* subdivided as in (*4*). From these wedge-shaped fragments cuboidal cortical explants are cut either with (*5, I*) or without (*5, II*) a part of the germinal epithelium.

CULTURE TECHNIQUE

Four to 5 fragments are placed on the top of a rather weak clot in an embryologic watch glass (Fig. 1, *1*) immediately after onset of coagulation. The medium most useful for this purpose is composed of 10–20% human blood plasma, 10% placental vein serum, 60–50% Gey's balanced saline solution and 20% human fetal brain press juice prepared as already described. The cultures are transferred onto another medium twice a week.

Behavior of the cultures.—As a rule the explants of the ovarian poles, at least in the ovaries obtained from older fetuses, behave better than those prepared from the body of the ovary. The explants without a part of the germinal epithelium attached to them generally degenerate very soon. However, around fragments containing a part of the germinal epithelium, the clot in the immediate neighborhood becomes liquefied and so the fragments float in a little pool of fluid. This condition favors epithelialization of the explants within 2 days.

The promotion of such epithelial enclosing membranes permits the cultivation of excised pieces of tissue, so that the techniques need not be limited to use of small organs having natural connective tissue or "epithelial" encapsulations.

Generally, in explants from fetuses of 4–6 months the parenchyma in the interior gradually disintegrates. However, in explants from 6–9 month fetuses the follicular cells of the pre-existing primordial follicles resist the degeneration process and clump together after the oocytes have died. During the following days the débris of the degenerating cells disappears. Meanwhile, cordlike proliferations start to grow from the covering epithelium toward the center of the explants. These cords soon anastomose and within them numerous oogonia develop in all stages of the prophase of the first maturation division. Some days later other epithelial cells in the same cords can form follicle cells, and in this way primordial follicles develop. These findings (5–7) lead to the conclusion that the covering epithelium of the explants apparently is of vital importance and is potentially capable of forming complete primordial follicles. The clusters of follicle cells in the explants made from the ovarian cortex of the older fetuses can also start proliferation, and in the cords thus formed again oogonia develop, which strongly indicates the intimate relationship of the cells of the germinal epithelium, the oogonia and the follicle cells.

REFERENCES

1. Erdmann, R.: Verwandtschaftsbeziehungen der Anurenfamilien, geprüft durch Implantationsversuche gezüchteter Haut, Arch. f. Entw. Mechanik d. Organ. 112: 739–806, 1927.
2. Gaillard, P. J.: Hormones Regulating Growth and Differentiation in Embryonic Explants, in Actualités scientifiques et industrielles (Paris: Hermann et Cie, 1942).

3. Gaillard, P. J.: Growth, differentiation and function of explants of some endocrine glands, Symp., Soc. f. exper. Biol. 2: 139-145, 1948.
4. Gaillard, P. J.: Transplantatie van gekweekte weefsels, Versl. afd. Nat Kong. Ned. Akad. v. Wetenschappen 58: 1, 1949.
5. Gaillard, P. J.: Germinal epithelium and sex cell formation, J. cyto-embryol. belgo-néerland. Gand., 1949.
6. Gaillard, P. J.: Sex cell formation in explants of the foetal human ovarian cortex: I, Proc. Royal Neth. Acad. Sc., Vol. 53, No. 8, 1950.
7. Gaillard, P. J.: Sex cell formation in explants of the foetal human ovarian cortex: II, Proc. Roy. Neth. Acad. Sc., Vol. 53, No. 9, 1950.
8. Gassul, R.: Experimentelle Studien über Auspflanzung, Ueberpflanzung und Regeneration von Explantaten aus erwachsenen Froschhaut, Arch. f. Entw. Mechanik d. Organ 52: 400–446, 1923.
9. Kooreman, P. J., and Gaillard, P. J.: Therapeutic possibilities of grafting cultivated embryonic tissues in man: I. The parathyroid gland in cases of postoperative tetany, Acta chir. Neerl., 2: 326–355, 1950.
10. Langman, J.: *Weefselkweek en transplantatie* (Amsterdam: Drukkerij Wed. G. Van Soest, 1950).
11. Stone, H. B.; Owings, J. C., and Gey, G. O.: Transplantation of living grafts of thyroid and parathyroid glands, Ann. Surg. 100: 613-628, 1934.

ACTION OF PHYSICAL AGENTS ON LIVING CELLS

Radioactive Tracers in Tissue Culture

AUSTIN M. BRUES *and* AGNES N. STROUD, *Argonne National Laboratory*

MANY POSSIBLE uses of tracers to study cell physiology in tissue cultures can be imagined, but most of these have not yet been realized. It is necessary to bear in mind certain limitations of existing tissue culture methods as well as limitations in the usefulness of radioisotopes depending on the physical nature of the isotopic radiation and the specific activities which can be obtained.

General procedures for which tracers are useful.—These include measurement of tracer concentration in various chemical forms in various tissues after injection into an animal, by analysis of the tissues of interest. For satisfactory interpretation of results in terms of metabolic processes, it is generally desirable to determine *specific activities* in all instances (a specific activity is defined here as the ratio of the radioelement to the corresponding normal element), which entails determination of the concentration of the normal element as well as of the radioelement in each analysis. Thus, after a culture of cells (1), or a suspension of erythrocytes, has come into equilibrium with K^{42} introduced into the medium, the intracellular K^{42} concentration will be much higher than the extracellular, while the specific activities are approximately equal. This means that reasonably complete mixing of all the K atoms in the system has been attained. Many biochemical reactions, e.g., the exchange of P between phosphates and phospholipids, appear to be essentially complete; while others, e.g., exchange of P with desoxyribonucleic acid, occur to a very slight extent (2). Synthesis is measured in the incorporation of radioiron into red blood cells during their formation.

Other applications include study of the fate of labeled compounds, such as drugs, and of trace elements (where, in many instances, specific activities are not determined); measurement of total body content of a readily diffusing or exchanging substance (as K^+ or Na^+ ions, or $H_2{}^3O$ as a tracer for water), and direct measurement of rate of blood flow or of the circulation rate through an area of vascular damage.

In certain instances pertinent isotopic measurements can be made on the totally intact system, as when the accumulation of radioiodine in the thyroid is measured externally. On the tissue or cellular level, such observations are possible during continuous cultivation in vitro (20).

Tissue cultures have certain advantages, in tracer studies, over tissue slices and whole animal experimental techniques. Tissues can be maintained continuously; growth and nutritive factors are under constant control, and morphologic studies can be carried on alongside serial determinations of tracer behavior.

Methods of measurement of radioisotopes are amply described elsewhere. Those in commonest use employ the electroscope and the Geiger-Müller counter, the latter activating either a scaler, which counts ionizing impulses, or a counting rate meter, which indicates the integrated counting rate. When great sensitivity is not required, a Lauritsen electroscope fitted with a thin window matching the culture bottle cover slip has been satisfactory.

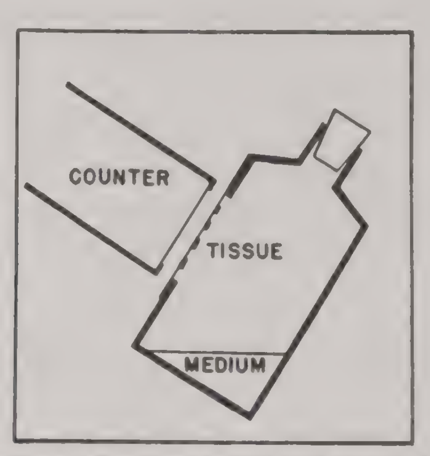

FIG. 1.—Relative positions of culture bottle and Geiger-Müller counter while readings of tissue radioactivity are being made.

Standard geometry is essential; i.e., the material being measured must be in such form and position that precise comparison may be made between samples and standards. A rack so constructed that the culture is centered under and at a constant distance from the electroscope or counter window is therefore necessary. When cultures are grown on a flat cover slip on a roller bottle (17), the uptake of isotope by tissue is measured directly in the position shown in Figure 1 (3). In the case of an isotope emitting a hard beta ray, such as P^{32}, K^{42} or Na^{24}, penetration of a glass cover slip is satisfactory and the isotope at the position of the tissue will give a much higher count than an equivalent amount in the medium due to absorption of beta rays in the relatively deep fluid medium and to the relatively more efficient position of the tissue, from which nearly half the beta rays will emerge in the direction of the window of the tube. Using a pure gamma ray emitter, the absorption of radiation in the fluid is negligible, leaving a difference due to the geometric factor alone.

Certain weak beta emitters (C^{14} and S^{35}) are of great interest biologically, but the low energy of the beta ray is such that penetration of a glass cover slip as used in tissue culture is minimal or zero. The thickness of a no. 1 cover slip is about 35 mg/cm², while the range of a C^{14} beta ray is about 20 mg/cm². It is possible to estimate C^{14} uptake by use of a thin Nylon sheet as cover slip attached to a roller bottle (thickness about 1 mg/cm²) (3), which is coated with plasma for explantation. The window of the electroscope or Geiger tube must also be very thin when these isotopes are used. In this case it is essential to remember that the *self-absorption of beta rays* in tissue, i.e., the fraction of the beta ray energy which does not emerge from the specimen, is likewise high, so that a relatively small proportion of the radioactive material in a tissue thickness of 100μ is counted, and there is a considerable selection factor in favor of the tissue nearest the cover slip. In the case of C^{14}, especially when it is introduced as bicarbonate or as a labile organic compound, $C^{14}O_2$ will appear in the gas phase and is much more efficiently counted than that in the medium. Since the ratio of $C^{14}O_2$:$HC^{14}O_3$ is dependent on the shifting pH of the medium, it is impractical to use direct readings except on washed cultures.

Autoradiography.—This may be used to determine the localization of an isotope in a culture. In the case of C^{14}, this was done by drying a washed culture on Nylon film, which was then stretched over photographic film in a dark room and clamped between glass slides for the required length of time (Fig. 2) (4). The optimal exposure time for an autograph must be determined by the experimenter for the particular film and experimental conditions. The specimens should be counted in close proximity to a Geiger-Müller tube, and with sensitive film a good autograph will be obtained in the length of time calculated to give about 10^6 counts/cm² of specimen (19). The autoradiographic method is useful in conjunction with other measuring techniques when it is desirable to make certain an isotope is actually incorporated in explants.

Localization of weak beta emitters or alpha emitters can be done on the cellular level by use of nuclear track emulsions (1). It must be remembered that fixation of tissues involves loss of water-soluble and other constituents. A method of preparing tissues without loss of water-soluble P^{32} has been described (12). We have successfully made autographs of frozen tissues through Nylon film (5).

Estimation of permeability rate.—When a substance is present in tissue at a significantly higher concentration than in the medium, it is feasible to measure its penetration into cultivated cells with the medium still present, in the way diagrammed in Figure 1. The calculations used in interpreting data have been published (7) and will be outlined here. In the interpretation of measurements in terms of permeability and metabolic turnover, it is necessary to know the concentration of the tracer and of the homologous element in both medium and tissue. The apparent radioactivity of

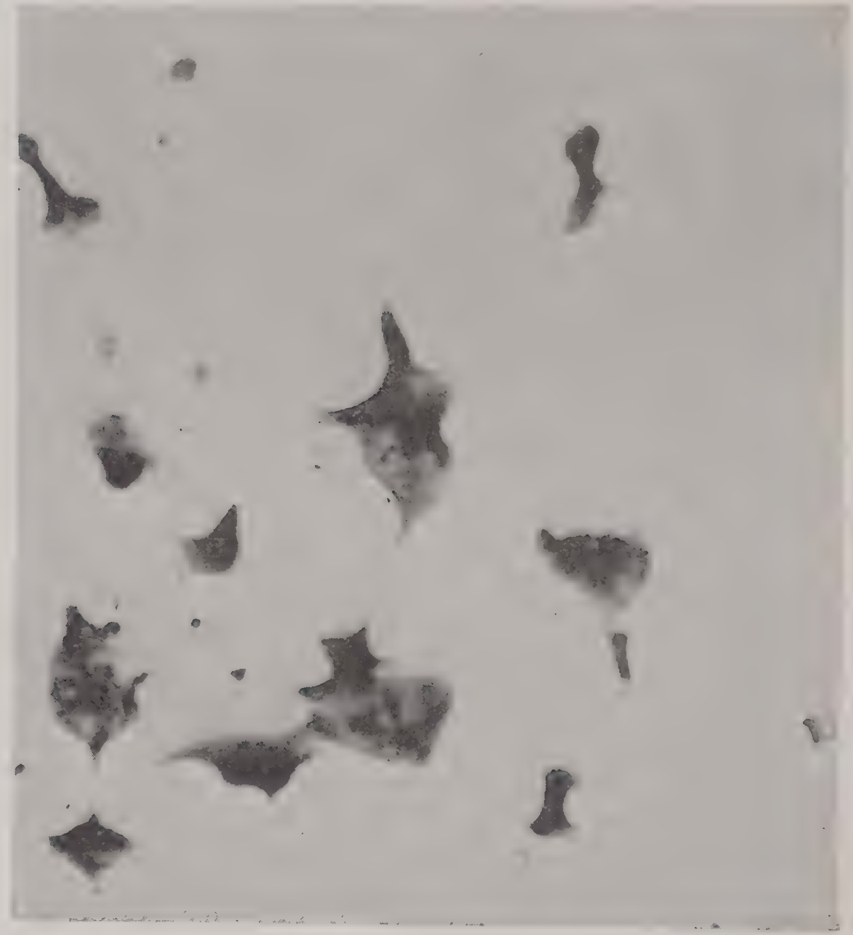

FIG. 2.—Autoradiograph of C^{14} incorporated into chick embryo heart tissue. Culture was grown in medium with $NaHC^{14}O_3$ for 3 days. Following several washings and fixation, exposure was made through the Nylon cover slip on Eastman nonscreen photographic film.

the culture (R) read on the counter can be translated into absolute isotope content of the culture (P'_i), using the initial count on the whole culture before the tissue has taken up the isotope (Co), a final count on the culture (R_f) and an absolute measurement of cell radioactivity (P'_{if}) made on tissue removed at the time of the final count. The true cell radioactivity then is related to interval counts by the equation

$$P'_i = \frac{P'_{if}}{R_f - Co}(R - Co)$$

Errors will be introduced if cultured fragments are thick with respect to the range of the radioactivity in question. Using P^{32} on tissue explants, this error is unimportant.

In an alternative method of measuring isotope uptake directly (13), counts are made after the medium has been drained into a side-arm of the culture bottle shielded from the counter. With either method, it is important that reproducible draining of medium from tissue be attained before measurements are made, since a significant amount of medium will necessarily remain in contact with the tissue unless it is washed.

When an uptake curve is obtained, permeability may be approximated by measuring the slope of this line at its origin. This slope, representing a steady state rate of exchange between medium and cells, has the dimension $Kd = dP/dt$, where P is the amount of element being measured. This is calculated from the specific activity of the medium S_{eo}, before radioactivity has entered the cells, and the measured initial rate of uptake of the isotope (dP'_i/dt), by the equation

$$\frac{dP'_i}{dt} = Kd \cdot S_{eo} \text{ at } t = 0$$

where S_{eo} is the ratio of P' (radioelement) to P (total element) expressed in the same units (as μc and mg) used elsewhere.

Permeability can be translated into usual terms if the cell surface of the culture is known. We have assumed from measurements of spindle-shaped chick embryo cells that the surface/volume ratio falls between 0.3 and 1.0 $\mu^2:\mu^3$, or between 3 and 10 cm^2/mg wet weight of living cells. Thus, Kd is divided by the estimated cell surface area.

Further analysis of uptake curves can be done when a single process will not account for the slope. This is accomplished through a semilogarithmic plot of the rate at which specific activities of the element in tissues and cells approach equality (7). Even when a simple exchange appears to take place, this may be the resultant of a more complex state of affairs, as in the case of K^{42} penetration into tissue which includes temperature-stable and temperature-unstable components. Figure 3 shows a semilogarithmic plot of equilibration of K^{42} with chick embryo tissue at 37 and 5 C (20). Curves showing loss of an isotope from a tissue which has been cultivated in the presence of an isotope may be used to demonstrate the reverse process, namely, loss of a substance in exchange for material taken in.

Since the mathematics of tracer behavior in complex systems is remarkably complicated, it is ordinarily necessary to perform analysis of tissue components in the study of metabolic processes. Hull and Kirk (13–15) have demonstrated the possibility of using P^{32} analysis of the nucleic acids in investigating metabolic factors in growth and a high correlation between growth rate and P^{32} uptake by desoxyribose nucleic acid of cultivated chick heart. Uptake of radioiodine by chick embryo thyroid has also been observed (16). When metabolic studies employing tracers do not require, or will not be facilitated by, continuous readings, any of the classic culture methods may be used. Cultivation should, as a rule, be done with a minimal amount of plasma.

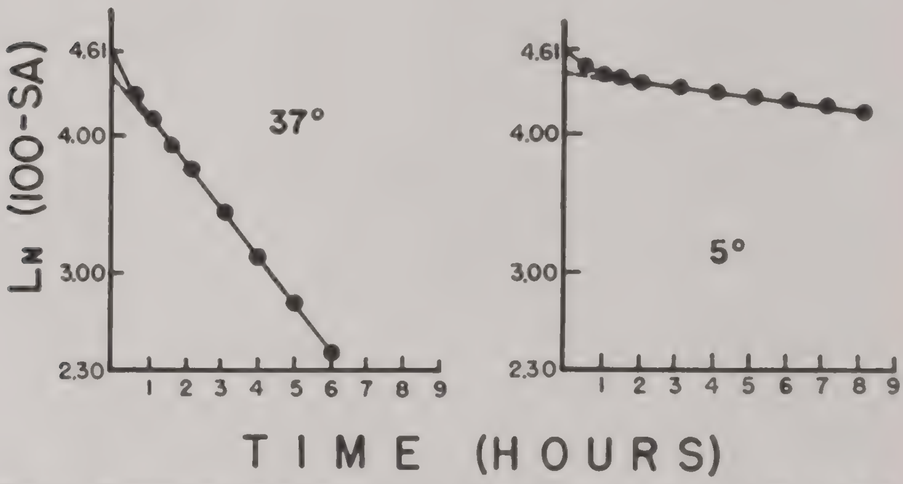

FIG. 3.—Logarithmic plots of uptake of K^{42} by tissue cultures at 37 and 5 C, showing presence of 2 components of K exchange.

For most tracer work quantitative culture methods will be essential. Our own techniques have been described (11) and additional pertinent techniques outlined by Signorotti, Hull and Kirk (18).

Other special applications.—When a tracer may be made to reach an equilibrium state between cells and medium, it can be used to estimate further shifts in the chemical element which it traces, provided the whole system (medium and tissue) is kept intact. In this connection, K^{42} was used to detect loss of K from cells to medium in cooled cultures, and the loss could be followed by external measurements of radioactivity (19).

P^{32} was used to estimate aliquot parts of divided explants (6). The cultures in question had taken up P^{32} in the course of cultivation and had then been washed to constant count with nonradioactive media. The separate fragments of the divided cultures were then measured under comparable conditions after subcultivation, giving an index of the respective mass of viable tissue in each fragment. By this method, 1 bit of tissue may be analyzed chemically or weighed as a basis of comparison.

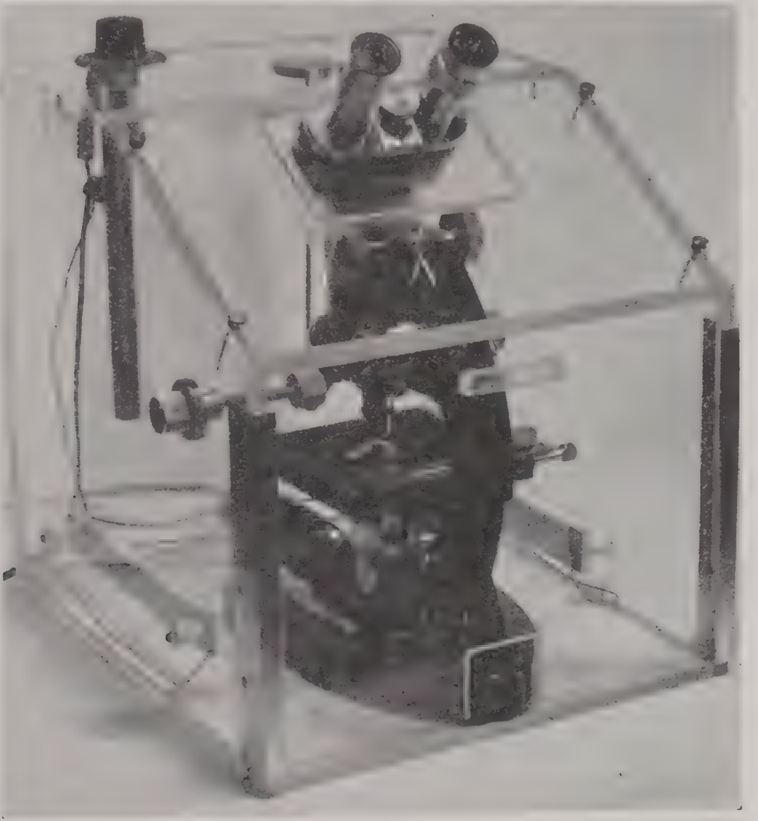

FIG. 4.—Microscope enclosed in a Lucite chamber kept at a constant temperature of 37 C.

The possibility of using tissue cultures in studying biosynthesis of labeled organic compounds should be mentioned because of its possible usefulness when it is desirable to use larger or better controlled incubators than is possible with tissue slices.

Radiation effects as applied to tracer experiments.—In the use of tracers one must be aware of the possibility that radiation effects may create abnormal conditions in a tissue. These responses are discussed by Buchsbaum (see pp. 255 ff.). Different cytochemical systems vary widely in their radiosensitivity; synthesis of nucleic acids (but not of proteins) may be impaired by dosages of a few hundred roentgen units (10), while tens of thousands are necessary to cause early death of appreciable numbers of resting cells in culture.

Calculations of the radiation dosage rate in a medium containing beta ray emitters are possible when the medium or mass of tissue is large with respect to the beta ray path. The following formula holds approximately: $60CE = D$, where D is dose in equivalent roentgens (rep) per day, C is the concentration in $\mu c/g$ and E is the mean beta energy in 10^6 electron volts (Mev). For 1 $\mu c/g$ P^{32} the dose is approximately 40 rep/day; in the case of C^{14} it is about 3 rep/day. The dose will be appreciably smaller when the culture is thin with respect to the beta ray path. Growth and uptake of P^{32} in tissue cultures have been observed at much higher concentrations of radioactivity (14). When doubt exists as to the validity of results from the standpoint of radiation damage, it is wise to perform an experiment in duplicate, varying the specific activity (and hence the radiation dose to cells) through a factor of 5 or 10 in 2 parallel experiments.

Radiation hazards.—These are controlled as in other isotope work. For observation, we have used a Lucite chamber (Fig. 4) impermeable to beta rays to hold a microscope. Volatile radioactive compounds (tritium oxide and $C^{14}O_2$) are handled in a hood during explantation and care is taken to keep the cultures sealed during incubation. The incubator should contain a dehydrating or CO_2-absorbing agent as an additional precaution.

REFERENCES

1. Boyd, G. A., *et al.*: Autoradiographs of C^{14} incorporated in individual blood cells, Science 108: 529–531, 1948.
2. Brues, A. M.; Tracy, M. M., and Cohn, W. E.: Nucleic acids of rat liver and hepatoma: Their metabolic turnover in relation to growth, J. Biol Chem. 155: 619–633, 1944.
3. Brues, A. M., and Naranjo, A.: Preliminary studies on C^{14} metabolism of tissue cultures, Anat. Rec. 100: 644, 1948.
4. Brues, A. M., and Buchanan, D. L.: Studies of the over-all CO_2 metabolism of tissues and total organisms, Cold Spring Harbor Symposia 13: 52–62, 1948.
5. Brues, A. M., and Stroud, A. N.: Unpublished data.
6. Brues, A. M., and Rathbun, E. N.: Unpublished data.
7. Cohn, W. E., and Brues, A. M.: Metabolism of tissue cultures: III. A

method for measuring the permeability of tissue cells to solutes, J. Gen. Physiol. 28: 449–461, 1945.

8. Curtiss, L. F.: Measurements of radioactivity, Nat. Bur. Stand. Circular no. 476, Oct. 15, 1949.
9. Evans, R. D., and Hamilton, J. G.: in Lawrence, J. H. (ed.): *Advances in Biological and Medical Physics* (New York: Academic Press, Inc., 1948), Vol. I, pp. 151–221.
10. Hevesy, G.: Effect of x-rays on the incorporation of carbon^{14} into desoxyribonucleic acid, Nature, London 163: 869–870, 1949.
11. Hildegard, W.; Jackson, E. B., and Brues, A. M.: The metabolism of tissue cultures: I. Preliminary studies on chick embryo, J. Gen. Physiol. 25: 689–703, 1942.
12. Holt, W. M.; Russell, F. C., and Warren, S.: Preparation of radioautographs of tissues without loss of water-soluble P^{32}, Science 110: 328–329, 1949.
13. Hull, W., and Kirk, P. L.: Tissue culture studies: IV. Growth of tissue in vitro as related to the direct measurement of P^{32} uptake, J. Gen. Physiol. 33: 343–348, 1950.
14. Hull, W., and Kirk, P. L.: Tissue culture studies: III. The relationship of incorporation of radioactive phosphorus in nucleic acid to the growth of cells in vitro, J. Gen. Physiol. 33: 335–342, 1950.
15. Hull, W., and Kirk, P. L.: Tissue culture studies: VI. The effect of medium constituents on nucleic acids and uptake of P^{32}, J. Gen. Physiol. 34: 81–85, 1950.
16. Rawson, R. W.: Personal communication.
17. Shaw, D. T.; Kingsland, L. C., and Brues, A. M.: A roller bottle tissue culture system, Science 91: 148–149, 1940.
18. Signorotti, B.; Hull, W., and Kirk, P. L.: Tissue culture studies: I. Standardization of techniques for quantitative culturing, J. Gen. Physiol. 33: 315–325, 1950.
19. Svihla, G.: Materials and Methods, in Bloom, W. A. (ed.): *Histopathology of Irradiation from External and Internal Sources*, National Nuclear Energy Series, Div. IV, 221 (New York: McGraw-Hill Book Company, Inc., 1949), pp. 22–24.
20. Wesson, L. G.; Cohn, W. E., and Brues, A. M.: The effect of temperature on potassium equilibria in chick embryo muscle, J. Gen. Physiol. 32: 511–523, 1949.

Methods of Studying Effects of Radiations on Tissue Cultures

RALPH BUCHSBAUM, *University of Pittsburgh*

The methods of tissue culture offer 2 main advantages over whole-animal material in studying the effects of any physical or chemical agent on biologic material: (1) the advantage of dealing with a group of cells of 1 type (or even a single cell) in a medium which may be known and controlled; (2) the advantage of direct observation. Of course, these advantages impose restrictions in drawing conclusions from experiments—the conclusions apply only to the experimental culture used. However, such conclusions may evolve hypotheses which may guide whole-body physiologic research.

Radiations, with the limitations described below, may be applied from a source external to the medium containing the cells or applied in solution in the culture medium as radioisotopes. The use of radioisotopes as tracers in tissue cultures is discussed in the foregoing article in this volume by Brues and Stroud. By an increase in concentration of the radioisotope well above the tracer level, investigations may be extended beyond the tracer application described by them to include various destructive actions on the tissue, cell or cell-organ in which the isotopes are concentrated.

We are concerned here with methods of studying radiations externally applied to cells or tissues in culture. Biologic research has been conducted on the effects of various relatively narrow parts of the electromagnetic spectrum from the longest radio to the shortest gamma rays. Duggar (4) and Lea (9) have given good general accounts. In general, the techniques are modifications of methods applied to whole-body radiation problems. The chief modifications of such methods are adaptations to microprocedures. For radio, heat, light, x-ray and gamma ray studies, the usual glass chambers (slides, tubes, flasks) present no special difficulties. Glass is relatively transparent to the whole spectrum except for the ultraviolet region. For ultraviolet work, quartz vessels are useful, or the irradiation may be made on uncovered cultures. Under these latter conditions it is obvious that bacterial contamination is improbable and that care must be taken to prevent loss of water from the culture.

In addition to irradiation of cultures with various wavelengths from the electromagnetic spectrum, cells may be bombarded with particles of different kinds: electrons, alpha particles, deuterons, protons, neutrons, etc. Here the chief problems are delivering the particles to the cells and knowing how many particles were received by the biologic object. Consideration must be given to the stopping power of the culture vessel and thickness of the overlying culture medium and of the cell preparation. Nuclear particles and electrons rarely have enough energy to penetrate a no. 1 cover slip as they leave the various sources available to the biologist. To overcome range difficulties, thin, strong and nontoxic materials such as mica and Nylon films may be used to carry the cells. The films should be 5 μ or less in thickness. Such films should be impervious to moisture (watch for minute cracks in mica and pinholes in Nylon); e.g., 5.2 Mev alpha particles from a Po^{210} source travel about 35 mm in air, about 35 μ in water and various intermediate distances in materials of higher atomic number than water. If the source is 3.5 mm from the culture chamber window, 10% of their path is used up. If a mica window 2 μ thick bears the culture, this path uses up an additional 10% of the total energy of the alpha particles, leaving approximately 80% of the original total path remaining for penetration through the medium into the cells. Since tissue is much like water in the composition of its atomic nuclei, this means that, under these conditions, the alpha particles cannot penetrate much more than 28 μ into the medium-plus-cells complex. On the other hand, hard beta particles from a P^{32} source can penetrate an ordinary tissue culture preparation.

The dose of radiation is determined by comparison with the electroscope, Geiger-Müller counter, scintillation counter, crystal counters with photomultiplier tubes and photographic films. A set-up can be made in which the beam of particles is counted through a dummy preparation without cells and this is compared with an experimental preparation, including cells. The difference represents the raw count of particles absorbed by the preparation. The instrument may then be calibrated, using known standards, to calculate the dose delivered to the preparation. Instruments are described in various places; for a bibliography on instrumentation see Pieper (11) and various issues of *Nucleonics* (10). Special consideration must be given to variations in the very thin layer of medium and tissue in a culture when calculating the energy absorbed. It is extremely important to standardize the application of irradiation because small differences in geometry may introduce serious variation into the experimental system. One must also bear in mind that the behavior of particles as they traverse the preparation varies with the type of particle. Beta particles produce relatively little ionization/particle/unit distance traversed, while alpha particles are densely ionizing. And the ionization is not uniform over the whole path. (See (13) with reference to specific ionization.)

The minimum dose to the culture may need to be increased 10-fold or more to accomplish effects in vitro that are observed in vivo. That this is probably related to the rate of renewal of the oxygen supply to the experimental culture seems to be demonstrated. In other words, if a tissue culture in vitro were given a supply of oxygen (and let me here add nutrients, waste removal, etc.) as adequate as that received by the same tissue in vivo, there is no theoretical reason now known which would lead us to expect a result in vitro different from that in vivo provided the absorption of the same amount of energy occurs in the same mass of tissue. For the foregoing statement to be true, it must be emphasized that proper precautions must be taken to insure that the few cells under observation do, in fact, receive the same amount of energy/unit mass in vitro as in vivo. For example, it is difficult to make a direct comparison between in vivo and in vitro experiments because it is difficult to equate the functional rate of flow of body fluids in vivo and the rate of exchange of materials in the tissue culture. Likewise, it is difficult to determine the equivalent masses of tissue being studied in the culture and in the intact animal.

Evidence of the biologic effects of radiation in tissue cultures may be gained from a comparison of irradiated with unirradiated control cultures in: rate of growth (3, 7), frequency of mitoses (6), damage to cells determined by morphologic changes (1, 5) or histochemical analysis. Here it may be cautioned that with very low dosage of irradiation an effect may not appear until after the preparation is in danger of deterioration and needs to be subcultured or the medium changed. Tissue cultures may be made of parts of irradiated whole animals to study tissue or cell changes which begin under conditions of whole-body irradiation. Also, the life of cultures under observation may be significantly prolonged by the use of perfusion techniques.

Small groups of cells, individual cells or parts of cells may be irradiated by various means. Tchakhotine (12) irradiated egg cells with ultraviolet rays focused to a 5 μ point and described local damage to the egg. Kisieleski, Svihla and Brues (8) developed a method of making minute beta emitters in the form of radioisotopes incorporated into glass beads. The beads may be placed above or within the culture medium. Buchsbaum and Zirkle (2) developed microbeams from 3 to 20 μ in diameter of 5.2 Mev alpha particles from a polonium source and irradiated parts of cells. Similarly, microbeams of protons, or other particles, may be developed for irradiation from other sources such as the Van de Graaff accelerator for the irradiation of even smaller parts of cells: centrioles, mitochondria, nucleoli, parts of chromosomes, etc.

REFERENCES

1. Bloom, W. A. (ed.): *Histopathology of Irradiation from External and Internal Sources*, National Nuclear Energy Series, Div. IV, 221 (New York: McGraw-Hill Book Company, Inc., 1949).
2. Buchsbaum, R., and Zirkle, R. E.: Shrinkage and swelling after alpha irradiation of various parts of large erythrocytes, Proc. Soc. Exper. Biol. & Med. 72: 27–29, 1949.
3. Doljanski, L., and Goldhaber, G.: Radiobiological studies on tissue cultures: I. The immediate effects of x-rays on cell outgrowth in cultures of fibroblasts, Growth 6: 235–249, 1942.
4. Duggar, B. M. (ed.): *Biological Effects of Radiation* (New York: McGraw-Hill Book Company, Inc., 1936).

5. Duryee, W. R.: The nature of radiation injury to amphibian cell nuclei, J. Nat. Cancer Inst. 10: 735–796, 1949.
6. Giese, A. C.: Radiations and cell division, Quart. Rev. Biol. 22: 253–282, 1947.
7. Halberstaedter, L.; Goldhaber, G., and Doljanski, L.: Comparative studies on the radiosensitivity of normal and malignant cells in cultures, Cancer Research 2: 20–31, 1942.
8. Kisieleski, W.; Svihla, G., and Brues, A. M.: Preparation of radioactive glass beads, Science 112: 400–402, 1950.
9. Lea, D. E.: *Actions of Radiations on Living Cells* (New York: Macmillan Company, Inc., 1947).
10. Nucleonics, Vol. 7, No. 5, 1950; also other numbers.
11. Pieper, G. F.: Instruments for radioactivity, Science 112: 377–381, 1950.
12. Tchakhotine, S.: Compt. rend. Soc. de biol. 120: 714, 1935.
13. Zirkle, R. E.: Relationships between chemical and biological effects of ionizing radiations, Radiology 52: 846–855, 1949.

ACTION OF CHEMICAL AGENTS ON LIVING CELLS

C. M. POMERAT

AMONG THE reasons for an increase in the use of tissue cultures for studies on the action of chemicals upon living cells are the following advantages:

1. Relatively low cost. In contrast to use of laboratory animals, the unit cost for tissue culture materials for assay purposes is much lower.

2. Rapid performance. Using simple hanging drop technique, 2 experienced workers easily can set up 600 test cultures a day.

3. Quick results. For toxicity studies, methods are available which provide results within 24–48 hr.

4. Genetic homogeneity. A large number of explants for a given experiment may be derived from a single donor animal. It is possible to use descendants from a single explant or even from a single cell (see pp. 217 ff.) for an unlimited number of studies.

5. Variety of test objects. Cultures may be prepared to represent all of the fundamental tissues, races of fibroblasts from various organs (51), malignant vs. corresponding nonmalignant cells, embryonic vs. adult tissues, as well as whole organs in vitro. Human tissues are uniquely available as test objects in culture since such objects as the buffy coat of blood, normal and pathologic tissues derived from surgical specimens and skin left over from grafting procedures are readily available in medical establishments.

6. Stringency of tests. The absence of vascular and nerve tissue as well as such detoxifying systems as the liver and the kidney reduces the number of variables involved in study of the action of chemicals on cells. The concentration of the test substance is not complicated by the variations in local concentration which develop in vivo. Physical factors are more easily controlled.

7. Emerging opportunities. Improvements in optical methods for study of the living cell, especially with phase contrast cinematography, fine structure with electron microscopy and the emerging possibility of having a medium for the animal cell of known chemical composition offer an important challenge to students of cell physiology and pharmacology.

No attempt will be made to present a comprehensive statement regarding the scope of this field, nor the variety of techniques described in the extensive literature dealing with the effect of chemicals on cells in vitro, but some general considerations will be outlined with illustrations of methods in current use.

I. GENERAL CONSIDERATIONS IN SELECTING TESTING METHODS

A. Use of Primary Explants vs. Cell Strains

C. M. POMERAT

1. *Primary explants.*—Observations on the outgrowth from tissue explanted directly from an embryo or adult organism offer the simplest and most inexpensive method of testing the effect of chemicals on cells. Its disadvantages include the fact that the relatively high percentage of cells which are injured or killed may serve to buffer against the agent being tested and otherwise influence the experiment. Factors of migration, cell division and readjustment to life in vitro are not as easily assessed as in well established cultures of cell strains.

2. *Cell strains.*—Many laboratories now have strains of normal and neoplastic cells which have been maintained in continuous culture for several years. Much information is available in the literature regarding the behavior of such strains.

Of special importance for these studies are the quantitative methods available for handling mass replicate cultures derived from an isolated individual cell, as described in this volume by Earle, Sanford and by Evans. With use of such material, effects resulting from the introduction of a chemical variable within the design of suitable controlled experiments afford excellent opportunities for systematic studies.

The disadvantage of the pure strain technique lies primarily in the extra cost of maintaining continuous cultures, especially in terms of the labor necessary to insure against contamination when handling cultures over long periods. Maintenance of cell strains during intervals between test experiments at 32 C or even lower temperatures reduces the frequency of replenishing the medium and thereby the cost of this operation (21).

B. Methods of Culturing

C. M. POMERAT

1. *Cover slip method.*—The lying or hanging drop method of culturing offers the simplest and least expensive procedure available which can be used primarily for large scale screening studies.

2. *Roller tube technique.*—The somewhat greater cost of this procedure over that of the cover slip method can be reduced by use of plain Pyrex test tubes without special constrictions and by use of a basic protein nutrient such as horse serum or human ascitic fluid which can be obtained in large quantities from individual patients with a minimum of technical work (69).

3. *Flasks for mass culturing.*—Carrel flasks and vessels of various sizes and shapes have been described for the study of metabolic products from large quantities of explants and for mass replicate culture (see pp. 218, 221 and 234).

4. *Watch glass methods.*—Little use has been made of the technique for cultivating whole organs (see this volume, pp. 237 and 241) for studies on the effect of chemicals on tissue complexes. Work in progress by Fell and Sir Edward Mellanby (personal communication) on the effect of high vitamin A dosage on bone formation indicates that this will afford an extremely valuable method for the analysis of chemical events in relation to complex morphogenetic phenomena under the highly controllable conditions of in vitro analysis.

5. *Special methods for phase contrast optics and electron microscopy.*—These are described on pages 284 ff. of this volume.

C. End-Points in Evaluating Effect of Chemicals on Cells

C. M. POMERAT

1. *Inhibition of outgrowth and death of cells.*—The simplest criterion for measuring toxic effects appears to be total inhibition of outgrowth from primary explants. In the face of the objection that this may occur occasionally in untreated control cultures due to trauma in handling or other technical complications, cessation of migration from a well established outgrowth can be determined, but this involves control procedures which are more costly in time and effort.

Workers dealing with the development of synthetic media have given much attention to the matter of survival of cells in vitro. The culture methods and end-points which they have evolved may be adapted with great profit in the study of drug effects under highly controllable conditions.

2. *Growth rate.*—An admirable account of methods for the measurement of growth rates is presented by Parker (52, Ch. XV). The use of nucleic acid estimations for quantitative studies on the

growth of cells in tissue culture is described by Waymouth in this volume (p. 225).

3. *Special criteria useful in evaluating chemical effects.*—Although no attempt will be made to classify and summarize the many endpoints utilized by workers employing tissue culture methods in the study of cytologic changes induced by drugs, a few examples are listed to illustrate something of the scope and possibilities which are available. This section, therefore, should be looked upon as no more than a suggestion of possible investigative approaches.

a) Contraction of muscular elements.—Paff (46, 47) and his associates (48, 49) have utilized the contractile activity in explants of chick embryonic hearts for assay of digitalis as well as for studies on its pharmacodynamic action. The long continued spontaneous rhythmic activity of skeletal muscle in tissue culture has been used to study the role of ryanodine on the contractile mechanism (54).

Records of action currents arising from rhythmically contracting systems in tissue cultures of embryonic chick heart fragments (43, 44) offer a stimulating challenge to students of irritable mechanisms in animal tissues.

b) Characteristics of malignant cells.—Workers in the field of oncology who are concerned with drugs which may affect malignant cells will find particularly valuable Lewis (32, 33) and Ludford's (36) summaries of many years of observations on the cytologic characteristics of malignant vs. corresponding types of nonmalignant cells.

Malignant cells in vitro are characterized by abnormally large nuclei, enlarged nucleoli and increased granularity of the nucleoplasm. Mitochondria repeatedly have been reported to be finer, less refractile and more numerous than those of corresponding nonmalignant elements. Conspicuously active undulating membranes, abnormal reactions to vital dyes and, of course, mitotic aberrations are associated with cells derived from neoplastic tissue.

Using electron microscopy in the study of malignant cells in tissue culture, Porter and Thompson (62) have noted a greater quantity of granular components in the endoplasm, some of which are strung together in the form of spiraling or twisted strands. These components may be associated with the high content of RNA in the cytoplasm of rapidly dividing cells. The value of using tissue culture material in the study of viral tumor agent with electron microscopy has been demonstrated (6, 63). This technique has been used for an investigation on the etiology of Hodgkin's disease (29).

c) Mitosis.—Many workers have described disturbances in the mitotic process resulting from certain drugs. Excellent models for tissue culture studies employed for this purpose are found in reports on mitotic abnormalities produced by mustard gas (11, 12, 24). The classification of mitotic poisons into those affecting the *spindle* as against those active directly on *chromosomes* was discussed by Pierre Dustin and by Arthur Hughes at the meetings of the VIIth International Congress for Cell Biology (see reports of communications).

Differential counts of various phases of the mitotic process have been utilized in the analysis of various antimitotic agents (15-17).

d) Nucleoli.—Variations in nucleolar size and form as well as in vacuolar content as described by Lewis (35), and regularly observed in cells under conditions of varying metabolic activity, assume new importance in the light of current views advanced by Caspersson and his associates on the role of the nucleus in the synthesis of proteins.

e) Mitochondria.—The association of enzymes with mitochondria makes these bodies of prime interest in studies of the action of drugs on living cells. Changes in the form of mitochondria as a result of mechanical disturbances, the action of enzyme poisons, changes in osmotic pressure and other controllable variables invite studies, particularly with phase contrast cinematography. Workers utilizing such techniques frequently are asked to describe the energetics involved in the movement of the mitochondria and to determine whether these movements follow particular pathways in the cytoplasm and whether individual enzymes or groups of enzymes can be shown to be associated with particular mitochondria. Genetic studies attempting to define the role of the cytoplasm in heredity may be especially directed at mitochondria, although all formed bodies in the cytoplasm are suspect.

f) Pinocytosis.—The role of viscosity changes in the protoplasm during pinocytosis (cell drinking), locomotion and cell division as described in classic studies (cf. 34) has not been exploited by cell physiologists and pharmacologists concerned with such phenomena as surface tension and the colloidal-chemical state of protoplasm. There is great need, for example, for systematic studies using tissue culture methods employing cinematographic recordings on the mechanism of surface-active agents and on the effect of compounds which influence the clotting of blood (cf. Heilbrunn in Rep. VIIth Internat. Cong. Cell Biol.).

Comment by John J. Biesele

Tissue cultures are of use in the screening of agents for biologic activity toward mammalian or avian cells for 2 reasons. Besides their economy in the use of agents, they allow an estimate of the direct effect of the test drug. The detoxifying, localizing and excreting activities of the whole animal can be avoided. For this reason, and because the agent, either per se or by virture of a metabolic product of it appearing only in the whole animal, may prove more toxic to some vital organ of the host animal and thus to the host itself than to the cells used for screening in vitro, results with tissue culture cannot be transferred directly to the whole animal. Nevertheless, tissue culture screening procedures are of value in the search for compounds of biologic interest. With some added effort they can be made the basis for special studies of mitotic, metabolic, nutritive or other effects of the agents on cells of one type or another.

What tissues shall be used depends on the end in view. If the aim is to find agents selectively damaging to malignant cells, the procedure described above seems reasonable. If the object is to locate drugs that affect the central nervous system, plantings of embryonic spinal cord may be made (57).

It is generally declared desirable to use subcultures of tissues that have been maintained in culture for some while, so that the cells may have become adjusted to tissue culture conditions, the population have become more homogeneous, the growth more standardized (52, 67). However, Painter, Pomerat and Ezell (50) have successfully used explants of chick spinal cord direct from the embryo, and our work has made use of tissue direct from animals (45). Repeated tests of certain purines with tissues fresh from animals have given nearly identical results (2). Besides the convenience of using material available in relatively unlimited quantities direct from animals, there is another possible advantage in that normal cells long maintained in continuous culture have been observed to undergo neoplastic change (19). The possibility that cell strains kept long in culture might be considerably different from cells of their tissue of origin could conceivably affect the significance and interpretation of their reactions to drugs.

Choice of the culture method is largely arbitrary. The most important requirement is that good replicate cultures be readily provided. It can be shown, for instance, with the Thompson rat fibroblast strain in roller tubes, that the variance in growth between tubes is greater than the variance between cultures within the same tube (28). Hence, the culture vessel rather than the single culture among several within a vessel is perhaps more properly regarded as the experimental unit. Other things being equal, it would seem that larger vessels provide an advantage in that they can contain a greater quantity of medium, which can therefore be more exactly compounded and more accurately dosed with drug. However, the better optical conditions of some smaller vessels permit more exact observations. Recent changes in technique to improve observations include excision of part of the clot near the explant (13) and introduction of a bubble into the clot (68). Both of these make for spreading of cells on the glass surface with no interfering clot behind them. Increasing use is being made of phase contrast microscopy and time lapse cinephotomicrography combined with an inverted microscope (24). The disturbing effect of clot lysis can be avoided by heating the serum at 60 C for 30 min before use (22).

Definitive work on the nutrition of mammalian cells in vitro must wait on the development of a satisfactory basal synthetic medium (39). Work with agents of no direct consequence in nutrition themselves is also hampered by lack of a synthetic medium, because it is possible that our present media of body fluids and tissue extracts contain substances that antagonize or enhance the activity of various tested substances. This is not to say that work with antimetabolites and drugs carried on in one variation or another of the classic medium of plasma, embryo extract and serum is valueless. Studies with antimetabolites might give clearer results if the medium were diluted with balanced saline solution to near the limit of safety. For some studies, it is possible to eliminate the plasma from the medium by replacing it by perforated cellophane (10). Replacement of the other complex natural nutrients will await their complete analysis or a lucky hit-or-miss addition to an incomplete medium.

What end-point to use in assessing the biologic effectiveness of a compound in tissue culture depends largely on what is being sought in the screening and on the biologic interest of the investigator. "Screening" implies a rapid method, and some of the procedures mentioned later are too arduous to qualify. Besides estimations of morphologic damage to cells, whether seen grossly as in cell rounding, granulation and disintegration or more delicately (and at lower concentrations of drugs) as in mitotic disturbances, determinations of effect on growth are possible. Most crudely and most readily, the lowest concentration of agent at which no outgrowth whatever occurs from the explant can be established (50). Growth rate is difficult to measure satisfactorily in tissue cultures. Subject to many precautions (52), the areal outgrowth from an explant can be determined with projector and planimeter. With more difficulty, the weight of cultures can be ascertained (30). Chemical means can be used to measure amount of nucleic acid phosphorus (71). The incorporation of nutrilites carrying radioactive atoms into the cultured cells can be followed (4). Methods are under development for planting cultures with known numbers of cells, and later of determining extent of growth by freeing and counting the nuclei (66). The search for a successful synthetic medium is being conducted with a single criterion: length of time the cultured cells are maintained alive in good condition, whether they proliferate or not (14, 39, 70).

II. USE OF TISSUE CULTURES IN DRUG-TESTING OPERATIONS

A. Primary Outgrowth on Cover Slip Preparations

C. M. POMERAT

The obvious and most direct method of evaluating drug toxicity with use of tissue explants consists in incorporating the substance to be tested, at various concentrations, in a medium suitable for the support, migration and growth of cells in vitro and determining after a reasonable interval of incubation whether or not the test tissues (*a*) resemble those in untreated control cultures, (*b*) show slight damage—least injurious dose (LID), or (*c*) show complete inhibition of cellular outgrowth. The lowest concentration of a chemical producing total inhibition or the minimal inhibitory dose (MID) is easily determined. With hanging drop preparations, such assays can be performed and evaluated in large numbers by technicians familiar with basic tissue culture methods.

The value of toxicity studies based on the primary outgrowth in cover slip preparations, however, depends on a complete report of the exact procedure used in the performance of the tests. On the basis of Jacoby, Trowell and Willmer's (27) demonstration that mitotic activity depends on the concentration of embryonic extract and Gaillard's (18) studies on the effect of the age of embryos on undifferentiated vs. organized growth in tissue culture, Parker (52) has emphasized that investigators should give detailed descriptions of the source of the growth-promoting factor in their culture medium. Moreover, a statement should be made regarding the method of cutting or homogenizing the embryos, the temperature and time interval used during the extraction procedure.

Methods generally used for preparing tissue extracts are given by Parker (52) and Cameron (5).

Useful species of cells.—Favorite objects for assay purposes include fibroblast-like cells derived from explants of the ventricular wall of the heart, leg muscle or frontal bone from 10–12 day old chick embryos.

Transverse sections of intestine of 8–10 day chick embryos provide an abundant source of explants for production of rapidly migrating epithelial cells. Outgrowths of compact sheets of epithelial cells in a single layer are under considerable tension and therefore are easily disorganized within 48–96 hr as a result of mechanical disturbances resulting from sudden changes in temperature and liquefication of plasma clots. However, much useful information can be obtained within the first 24–48 hr of incubation. A low concentration of growth-promoting substances in the medium and, possibly, the adaptation of Morgan and Parker's (40) use of crude soybean antitryptic factor to prevent the digestion of the coagulum may be employed to enhance the value of explants of epithelial cell cultures from chick intestine.

Within a few hours of incubation at 37 C, 1 mm^2 fragments of embryonic chick spleen produce symmetrical radial outgrowths which reach a diameter of approximately 5 or 6 mm by the end of the first day. Spleens of 18–20 day chicks have been found particularly useful test objects (56, 59). The cell types in chick spleen and their behavior in vitro were described by Danchakoff (7) and Rioch (64).

Transverse sections of spinal cords from 9 day chick embryos have been utilized for testing the toxicity of central nervous system stimulants and depressants on the outgrowth of nerve fibers (50, 57). The method of obtaining explants from the spinal cord is shown in Figure 1.

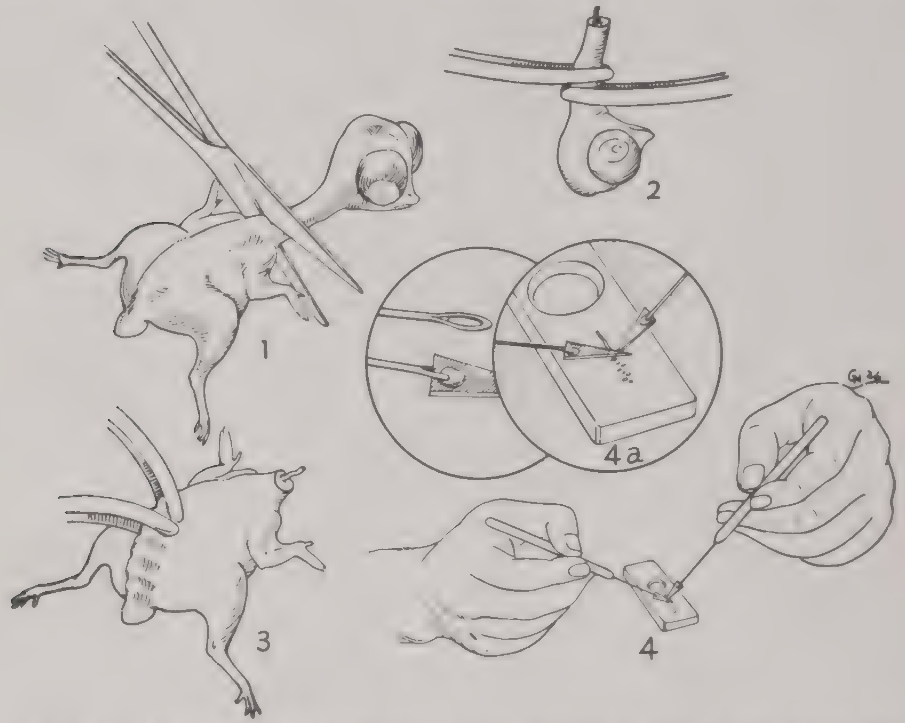

FIG. 1.—Method of obtaining spinal cord explants from 9 day chick embryos. Neck is severed (*1*) and cord is expressed from the spinal canal "like tooth paste" by means of forceps (*2*, *3*). The segments of spinal cord are cut into transverse sections (*4*, *4a*). *4a* shows method of making small knives by mounting wafer blade fragments into the broken eye of sewing needles with use of metal solder.

Beyond these readily available sources of fibroblastic, epithelial and nerve preparations from chick embryos, a wide spectrum of well established cellular test objects is suggested in the literature. Of growing importance to workers in the medical field is the use of adult human tissues obtained during surgical procedures. Although adult tissues have the disadvantage of usually showing a longer lag phase before the appearance of migrating cells, the objection that embryonic tissues have fundamental differences in metabolic activity and antigenicity, together with the matter of species specificity, can be overcome for investigations directed at the relation of chemicals affecting the protoplasm of *human* adults.

Fibroblast-like cells are readily obtained from subcutaneous, areolar or mesenteric tissue derived from healthy areas of surgical specimens. Tonsils free from active pyogenic processes yield satisfactory cultures of a wide variety of cellular elements when cultivated in medium containing antibiotics. Penicillin G, at concentrations of as much as 1000 units/ml of medium, other antibiotics and sulfonamides are being used successfully in the cultivation of various contaminated tissue explants. Lymph nodes provide an important source of human cells.

The buffy coat of human blood (8, 9, 42, 52) is a strikingly interesting object for study of individual differences in cell behavior which is obtainable without undue discomfort to donor subjects.

Skin left over from grafting procedures is an excellent source of epithelium for short term culture studies (3, 31, 69). Biopsies of mucous membrane from the middle turbinate yield sheets of pavement epithelium, often ciliated, which are suitable for work on histamine, antihistaminic drugs and specific antigens, in relation to well established allergies (65).

Successes in the cultivation of skeletal muscle from adult mammals (54) invite studies, particularly in relation with techniques for recording action currents from rhythmically contracting human adult fibers.

Ganglia from the lower thoracic and upper lumbar sympathetic trunks, as well as celiac, splanchnic and stellate ganglia obtained by surgical procedures generally directed toward the relief of essential hypertension and therefore showing no gross histologic abnormalities, make available extraordinarily valuable material for studies on adult nerve cells, their processes and associated elements (41). Lobotomies and similar procedures also provide challenging opportunities for the cultivation of cells from biopsies of adult human brain (58). Although such material removed from patients with a diagnosis of mental disorder is not proved free from demonstrable pathology, occasional operations for the relief of intractable pain resulting from malignancies and tissue obtained during débridement and ventriculography make available brain explants which may be expected to be less altered than those associated with intracranial tumors. Although much preliminary work must be done to define ideal conditions for the growth of nervous and glial elements, the demonstration of deposition of myelin in vitro (53) is of great importance in the study of demyelinating diseases and in relation to effects on nerve tissue.

PROCEDURE

Substances to be tested generally are sterilized by autoclaving or are dissolved in balanced saline solution at relatively strong concentrations and passed through a micro Seitz filter. From stock solutions, serial dilutions are made with balanced saline solution, then mixed in equal proportions with embryonic extract. Tuberculin syringes with truncated 27 gauge needles are useful in setting up hanging drop preparations. One drop of heparinized cockerel plasma is placed on the cover slip, the test explant is added, 1 drop of the drug-embryo extract combination is quickly mixed in to form the clot and the tissue is left in the center of the cover slip. Rapid, complete stirring is essential to insure uniform distribution of the components. Using this system, the final concentration of the chemical under study will be only $^1/_4$ the concentration of that in the drug-balanced saline solution dilution mixture.

In the initial screening process, 4 or 5 cover slip preparations are made for each of 5 dilutions in 1:10 steps. Subsequent checks and additional dilutions are carried out to the point desired within the objectives of the study.

After incubation, preparations are usually examined at 24 hr intervals unless stimulation is suspected, in which case it may be possible to detect outgrowth exceeding that of the controls during the early period of incubation.

The most satisfactory end-point for toxicity studies is the lowest concentration of the test substance required to produce total inhibition of outgrowth from the explant (MID). Somewhat more difficult to determine is the concentrate at which outgrowth is somewhat less than that found in corresponding control cultures (LID). The rating of outgrowth may be made at a suitable microscopic magnification by placing the edge of the explant on the rim of the ocular field and estimating whether the migrating elements extend across $^1/_4$, $^1/_2$, $^3/_4$ or the entire field. Arbitrary ratings of −, +, ++, +++ and ++++ are often used for tabulation of results. More accurate evaluations can be made by using an ocular micrometer and, especially, by drawing an outline of a projection of the outgrowth for analysis with planimetric and related methods. Growth measurements and their interpretation have been described by Parker (52).

When chick embryo tissues are utilized, final readings may be made at 24–48 hr for splenic, intestinal and spinal cord explants; explants producing fibroblast-like cells are read after 48–72 hr of incubation. Staining of culture preparations is useful for more detailed studies or for preservation of permanent records.

B. Medawar's Hanging Drop Assay Test

C. M. POMERAT

This method (37, 38) utilizes explants from the ventricle of 10 day chick embryos in 0.1 ml of equal parts of fowl plasma without anticoagulant and of balanced saline solution containing the drug to be tested. After 48 hr of contact with the drug, explants may be washed for 1 hr in balanced saline solution, then embedded in a plasma clot. Thus it may be possible to determine whether an initial toxic effect is irreversible or whether recovery permits subsequent outgrowth of cells.

C. Flask Method (Parker)

C. M. POMERAT

As suggested in the general remarks in this section, tests using established strains of cells in Carrel flasks or roller tubes probably increase the reliability of the results even though the cost of equipment and time for manipulation constitute an important drawback. Parker (52) considers flasks more suitable than roller tubes primarily because of the greater facility they offer for making observations, records and photographs. He recommends embedding the tissue in a purified fibrogen coagulum to standardize the conditions for culture.

D. Titration of Toxic or Lethal End-Points (Hanks)

C. M. POMERAT

Suitable tissues from a young and susceptible animal will furnish a large number of explants from a common source.

Procedure A Microscopic Evaluation	Procedure B Physiologic Evaluation
Take pooled explants up in 10% chicken plasma and transfer individual fragments to a position low on the walls of 13 mm test tubes. Discard all excess fluid.	Transfer a standard size or number of fragments to 13 mm test tubes in 5 drops of nutrient fluid (ultrafiltrate, etc.).
After 24–48 hr for migration, discard atypical colonies.	After preliminary incubation, discard tubes with atypical pH changes.
Add 10 drops of supernate containing standard nutrient back-	Same

ground and, as variables, the desired proportions of toxin vs. antitoxin, virus vs. antibody, etc.

Base microscopic conclusions on the proportion of cultures in which no cells appear to have survived.

Base conclusions on proportion of cultures in which the pH drop corresponds to that in the control group.

Titration end-points should be selected at such levels that conclusions may be drawn in an interval not requiring renewal of the supernatant fluids.

The large numbers of units which can be handled, the common source of the cells and the controlled uniformity of the cell environment suggest that tests developed along such lines could be exploited profitably in a variety of problems.

Hanks (23) considers this method valuable not only for the study of drugs but for observations on antibodies. It permits the discarding of explants not showing vigorous outgrowth before the test substance is applied. The method of physiologic evaluation by the simple expedient of considering that changes in pH provide the best single over-all measurement of metabolic activity deserves wider use.

E. Tissue Culture Screening of Agents in Experimental Cancer Chemotherapy

JOHN J. BIESELE, *Sloan-Kettering Institute*

The screening test is essentially one of comparing the toxicities of an agent to normal and malignant cells in vitro. It has the advantages of economy in use of agents available in only limited amounts and of direct application of the agents to the cells of interest. Although it was described by Ormsbee *et al.* (45), the description given here is a modification in use at the Sloan-Kettering Institute.

PROCEDURE

A transplantable tumor known to grow well in culture is taken at a time of vigorous growth from a host animal. Mouse tumors used for this purpose have included Crocker mouse sarcoma 180, sarcoma 241 of C57 black mice, carcinoma 1025 of AKm mice, and others. Under aseptic precautions, healthy portions of the tumor are cut into fragments 1 or 2 mm on a side and planted in a chicken plasma clot in a roller tube, 6 fragments being placed in a row.

Normal tissues for comparison are taken from newborn mice of the same strain as that carrying the tumor or from embryonic mice. Tissues routinely used are the heart and especially the belly skin of embryos of about 14 days' gestation. These are also cut into pieces a few mm on a side and planted in rows of 6 in the same tubes with the tumor explants. The screening tube thus carries 6 explants of a tumor and 6 explants of newborn or embryonic tissue.

Fluid nutrient medium is added to the roller tubes, 1 ml/tube. The medium is composed of 4 parts Gey's balanced saline solution, 2 parts 10 day chick embryo extract (equal parts embryo and Gey's solution), 1 part human placental serum and 3 parts horse serum. The initial pH is 7.4–7.8. The medium also contains 25 units of penicillin and 25 units of streptomycin/ml.

The roller tube cultures are incubated at 37 C for 1 day in a drum rotating 10 times/hr. The cultures are then examined at a magnification of ×100, and extent of outgrowth and general condition of the cells are noted. Tubes showing poor growth or unhealthy cultures are discarded. The cells of interest have customarily been sarcomatous cells, on the one hand, and fibroblast-like cells growing out from the embryonic tissues, on the other.

Agents to be tested are then applied to the cultures; 0.1 ml of the medium is removed and replaced by a like volume of solution or suspension of the agent in sterile saline or other suitable vehicle. To safeguard sterility, the dry agent may be heat-sterilized if stable, or solutions of the agent may be passed through bacteriologic filters or be boiled if sufficiently stable. Three or 4 concentrations of agent are used, with 2–10-fold differences between successive concentrations. Two or 3 roller tubes are used for each concentration. Several control tubes are given replacement volumes of 0.1 ml of the vehicle alone.

After an additional day of incubation in presence of the agent, the cultures are again examined microscopically and the extent of morphologic damage to the sarcomatous and embryonic cells is assessed. Damage is graded as extensive, moderate, slight or absent on the basis of retraction of cytoplasmic processes, increased protoplasmic granularity, cellular disintegration, inhibition of growth and proportion of cells showing the morbid changes. The grading depends on the observer's experience with the reaction of the cells in question to damaging influences. Comparison is made with the condition of the control cultures at the same time.

Comment.—It is at times desirable to carry out additional experiments with an agent in order to reach the lower limits of toxic concentration for normal or malignant cells or to establish more precisely a selectively damaging concentration.

Test procedures of this sort contain many arbitrary elements.

Variations are sometimes introduced. For example, in tests of the steroid hormones, Kendall's compounds E and F, it was found desirable to include the hormones with the initial medium and to add fresh hormone when the cultures were refed on the third and sixth days, in order to demonstrate greater injury to the cells of certain sarcomas after a week or more of cultivation (1). The routine 48 hr test has been sufficient to make differences in the effects of such substances as purines evident (2).

The screening test may, with slight modification, be used in study of metabolite-antimetabolite relationships. This is possible despite the necessity of using a nonsynthetic medium of uncertain constitution, if the suspected antimetabolite is injurious when used alone. It is only necessary to introduce the suspected normal metabolite in a series of concentrations together with the antimetabolite in a known toxic concentration. By observing the blocking of damage, inhibition ratios may be determined. It has thus been determined that the agent 2,6-diaminopurine, which injures cells of sarcoma 180 in vitro at a considerably lower concentration than that at which it damages embryonic mouse fibroblasts, is blocked more readily by adenine in the case of embryonic fibroblasts than in the case of sarcomatous cells (2).

Preparations for more careful cytologic studies may be made by planting on cover slip inserts in flattened roller tubes. Such preparations, when fixed and stained, serve as permanent records and allow a study of such matters as the induction of mitotic abnormalities to be made. For the latter studies, it is usually necessary to use concentrations of agents considerably lower than those causing the gross damage noted in the screening tests.

Comment by C. M. Pomerat

The objection of undesirable optical conditions in the roller tube technique is met, in part, by Porter, Claude and Fullam's culture vessel (61 adapted for a roller tube rack and also by use of narrow cover glasses o no. 1 thinness introduced in the interior of test tubes. Cover glass prepara tions cannot be easily examined through the walls of roller tubes except a very low magnification, but in some types of experiments satisfactory results are obtained by removing cover slips for examination at regular intervals.

A roller tube technique being evolved for the study of cells from human pleural and peritoneal exudates (25, 26, 60) may prove particularly usefu to workers in the medical field. Spindle cell strains, probably derived from mesothelial cells, histiocytes and possibly from monocytes in ascitic flui from nonmalignant processes such as congestive heart failure and cirrhos of the liver, can be obtained in large numbers of cultures. Reasonably reproducible results may be developed by estimating the density of th "seeding" with an adaptation of techniques such as those described by Evans (p. 221) for the handling of replicate mass cultures. The effect of various physical and chemical variables on neoplastic cells from malignant pleural and ascitic fluid may be explored by use of the vast quantities of such material available in hospital centers.

Successful adhesion of explants or individual cells to clean glass surfaces, as utilized by workers testing the merit of synthetic media, should be employed whenever possible so that test chemicals subsequently added will come in direct contact with the cells. As in nutritional studies, it is especially important to avoid plasma clots in experiments involving substances having high molecular weight and also those with low solubility, such as the sex hormones.

III. CYTOCHEMICAL STUDIES

Perfusion Chamber

C. M. POMERAT

A metal frame and the assembly technique suitable for perfusion of cells in tissue culture are shown in Figure 1. Aluminum or, better, stainless steel is used for constructing the metal frame. Noncorrosive cover glasses are used both for the floor of the chamber and as a surface for growth of the culture. Inlet and outlet perfusion tubes are made of Pyrex glass. A mixture of equal parts of beeswax and paraffin (mp 50°) is used to attach the cover glasses and perfusion tubes to the metal frame. Dimensions of component parts of the set-up are given in the diagram.

Special care must be used in preparing capillary tubes of suitable diameter, their size depending on the optical system used for observations. The working distance between the top of the substage condenser and the objective as well as the desired rate of flow through the chamber are determining factors following the design of particular experiments. A C- or S-shaped bend at the free end of the outflow tube is desirable to prevent emptying of the chamber containing the living cells. When filling the chamber it is important to avoid air bubbles in the tube and in the space between the cover glasses. Filling is facilitated by holding the outlet end of the metal frame elevated and rocking the slide so as to encourage an even spreading of the fluid in the interior of the perfusion chamber.

The perfusion chamber is easily set up under aseptic conditions. In certain types of experiments antibiotics may be added to the perfusion fluid to insure against chance contamination. Cover slip preparations from hanging or Maximow flying drop preparations are well suited for this type of study. Cover glasses measuring

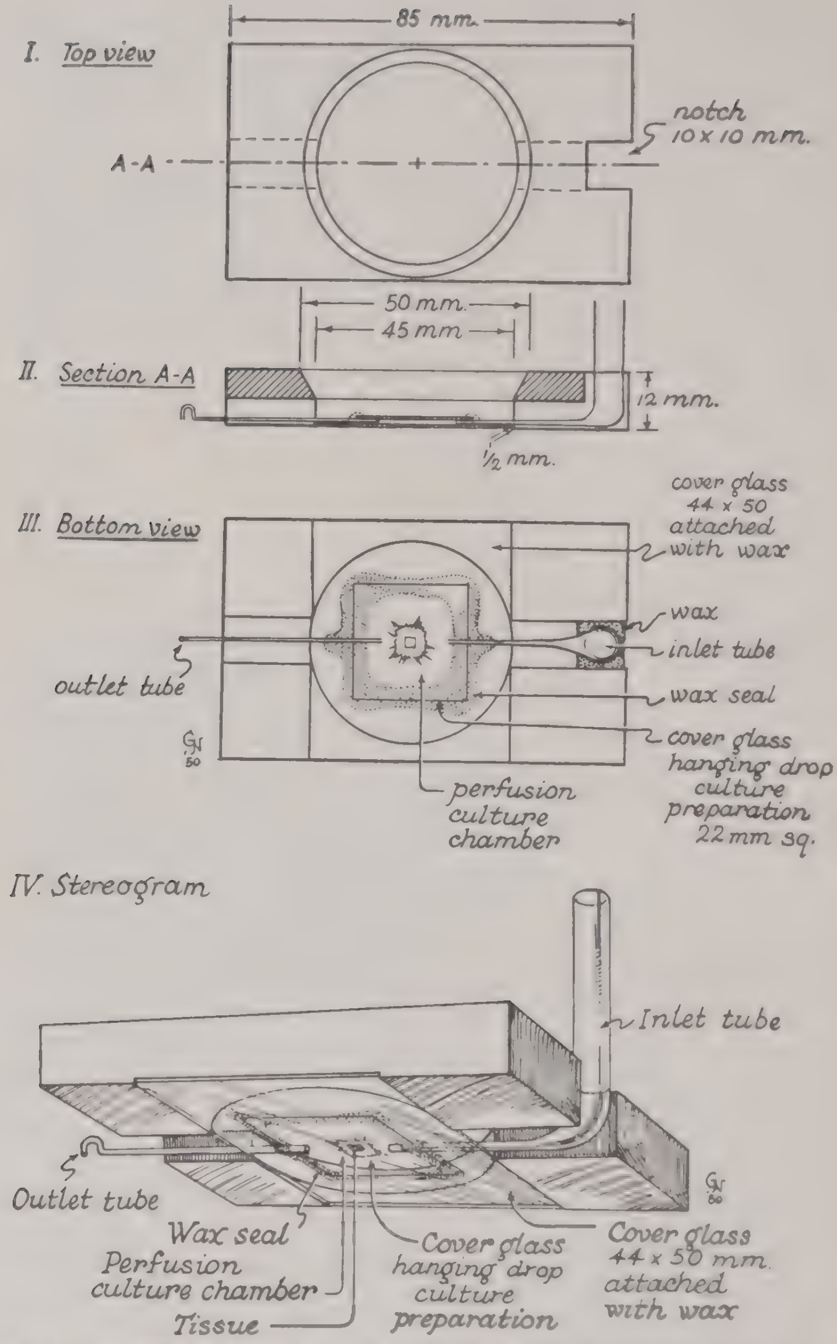
I. Top view
85 mm.
notch
10 x 10 mm.
A-A
50 mm.
45 mm
II. Section A-A
12 mm.
½ mm.
III. Bottom view
cover glass
44 x 50
attached
with wax
wax
inlet tube
outlet tube
wax seal
cover glass
hanging drop
culture
preparation
22 mm sq.
perfusion
culture
chamber
GN
'50
IV. Stereogram
Inlet tube
Outlet tube
Wax seal
Perfusion
culture chamber
Tissue
Cover glass
hanging drop
culture
preparation
Cover glass
44 x 50 mm.
attached
with wax
GN
'50

FIG. 1.—Diagram of metal frame for a perfusion chamber. *I*, top view; *II*, section *A–A*; *III*, bottom view; *IV*, stereogram showing arrangement of the culture in relation to inlet and outlet tubes for perfusion of fluid medium.

12×50 mm on which 2 explants are attached and cultivated in test tubes have the advantages of the roller tube method and, for examination, the glass may be broken across the middle of its long axis, 1 culture being fixed as a control while the other is mounted in the perfusion system. The medium in which the cultures were grown may be used, at least, as the initial perfusate. Fluid for perfusion should be warmed to the same temperature as that of the rest of the system.

This perfusion system permits cinematographic recordings with phase contrast objectives under oil immersion. Methodologically, prime consideration must be given the establishing of reproducible base lines before recording the effects of physical and chemical agents on the activity of the margin of cells, the mitotic process or the behavior of cytoplasmic organells.

REFERENCES

1. Biesele, J. J.: Effect of Compounds E and F on Normal and Neoplastic Mouse Tissues in Culture, in *Conference on ACTH and Adrenocortical Steroids* (New York: American Cancer Society, 1951).
2. Biesele, J. J., *et al.*: Studies on 2,6-diaminopurine and related substances in cultures of embryonic and sarcomatous rodent tissues, Cancer 4: 186–197, 1951.
3. Blocker, T. G., Jr.; Pomerat, C. M., and Lewis, S. R.: Research opportunities with the use of cultures of living skin, Plast. & Reconstruct. Surg. 5: 283–288, 1950.
4. Brues, A. M.; Rathbun, E. N., and Cohn, W. E.: Metabolism of tissue cultures: II. Estimation of total growth in tissue culture; growth in incomplete media as a "steady state," J. Cell. & Comp. Physiol. 24: 155–158, 1944.
5. Cameron, G.: *Tissue Culture Technique* (New York: Academic Press, Inc., 1950).
6. Claude, A.; Porter, K. R., and Pickels, E. G.: Electron microscope study of chicken tumor cells, Cancer Research 7: 421–430, 1947.
7. Danchakoff, V.: Equivalence of different hematopoietic anlages (By method of stimulation of their stem cells): I. Spleen, Am. J. Anat. 20: 255–328, 1916.
8. Draper, G.; Pierce, C., and Dupertuis, C. W.: The relation between cells and plasma in cultures of the buffy coat from human blood, Am. J. M. Sc. 210: 738–745, 1945.
9. Draper, G.; Ramsey, H. J., and Dupertuis, C. W.: Variation in behavior of buffy coat cultures among individuals of different constitutional types, J. Clin. Investigation 23: 864–874, 1944.
10. Evans, V. J., and Earle, W. R.: The use of perforated cellophane for the growth of cells in tissue culture, J. Nat. Cancer Inst. 8: 103–119, 1947.
11. Fell, H. B., and Allsopp, C. B.: The action of mustard gas ($\beta\beta'$dichlorodiethylsulphide) on living cells in vitro, Cancer Research 8: 145–161, 1948.
12. Fell, H. B., and Allsopp, C. B.: The effect of repeated applications of minute quantities of mustard gas ($\beta\beta'$dichlorodiethylsulphide) on the skin of mice, Cancer Research 8: 177–181, 1948.

13. Fell, H. B., and Hughes, A. F. W.: Mitosis in the mouse: A study of living and fixed cells in tissue culture, Quart. J. Micr. Sc. 90: 355–380, 1949.
14. Fischer, A.: Morphological aspects of animal tissue cells in synthetic media, Acta Anat. 5: 57, 1948.
15. Friedmann, E.; Marrian, D. H., and Simon-Reuss, I.: Analysis of antimitotic action of certain quinones, Brit. J. Pharmacol. 3: 263–270, 1948.
16. Friedmann, E.; Marrian, D. H., and Simon-Reuss, I.: Sulphydryl addition compounds of some quinones and related substances in their action on the growth of normal cells, Brit. J. Pharmacol. 3: 335–340, 1948.
17. Friedmann, E.; Marrian, D. H., and Simon-Reuss I.: Antimitotic action of maleimide and related substances, Brit. J. Pharmacol. 4: 105–108, 1949.
18. Gaillard, P. J.: Growth, differentiation and function of explants of some endocrine glands, Symp. Soc. Exper. Biol. 2: 139–145, 1948.
19. Gey, G. O.: Cytological and cultural observations on transplantable rat sarcomata produced by the inoculation of altered normal cells maintained in continuous culture, Cancer Research 1: 737, 1941.
20. Gey, G. O.: Personal communication, 1950.
21. Gey, G. O.; Hanks, J. H., and Barrett, R.: Retardation of growth and metabolism of normal and malignant cells during cultivation, Growth 12: 69–105, 1948.
22. Goldhaber, P.; Cornman, I., and Ormsbee, R. A.: Experimental alteration of the ability of tumor cells to lyse plasma clots in vitro, Proc. Soc. Exper. Biol. & Med. 66: 590–595, 1947.
23. Hanks, J. B.: Lecture data, Tissue Culture Course, Univ. of Toronto, 1948.
24. Hughes, A. F. W., and Fell, H. B.: Studies on abnormal mitosis inducted in chick tissue cultures by mustard gas ($\beta\beta'$dichlorodiethylsulphide), Quart. J. Micr. Sc. 90: 37–55, 1949.
25. Ivers, J. B., and Pomerat, C. M.: Observations on cells from ascitic fluid cultured in vitro, Texas Rep. Biol. & Med. 5: 92–111, 1947.
26. Ivers, J. B.; Pomerat, C. M., and Neidhardt, H. W.: Hormonal relations to neoplastic growth with report of a case study, Texas Rep. Biol. & Med. 6: 206–233, 1948.
27. Jacoby, F.; Trowell, O. A., and Willmer, E. N.: Studies on the growth of tissues in vitro: V. Further observations on the manner in which cell division of chick fibroblasts is affected by embryo tissue juice, J. Exper. Biol. 14: 255–266, 1937.
28. Jacquez, J. A.: Personal communication.
29. Jacquez, J. A., and Porter, K. R.: Electron microscopy observations on Hodgkin's material, Cancer 2: 853–856, 1949.
30. Laser, H.: Flächengrösse und Wachstum von Gewebekulturen, Ztschr. f. Krebsforsch. 39: 384–390, 1933.
31. Lewis, S. R.; Pomerat, C. M., and Ezell, D.: Human epidermal cells observed in tissue culture with phase-contrast microscopy, Anat. Rec. 104: 487–503, 1949.
32. Lewis, W. H.: Malignant cells, Harvey Lect. 31: 214–234, 1936.
33. Lewis, W. H.: Some cultural and cytological characteristics of normal and malignant cells in vitro, Arch. f. exper. Zellforsch. 23: 8–26, 1939.
34. Lewis, W. H.: The relation of the viscosity changes of protoplasm to ameboid locomotion and cell division, in *A Symposium on the Structure of Protoplasm* (Ames, Ia.: Iowa State College Press, 1942), pp. 163–197.
35. Lewis, W. H.: Nucleolar vacuoles in living normal and malignant fibroblasts, Cancer Research 3: 531–536, 1943.

36. Ludford, R. J.: Structure and behavior of the cells in tissue cultures of tumors, 11th Sci. Rep. Imp. Cancer Research Fund, pp. 147–168, 1934.
37. Medawar, P. B.: A factor inhibiting the growth of mesenchyme, Quart. J. Exper. Physiol. 27: 147–160, 1937.
38. Medawar, P. B.: The growth, growth energy, and ageing of the chicken's heart, Proc. Roy. Soc., London, s.B. 129: 332–355, 1940.
39. Morgan, J. F.: Development of Synthetic Media, in Parker (52), Ch. VIII, pp. 115–131.
40. Morgan, J. F., and Parker, R. C.: The use of antitryptic agents in tissue culture: I. Crude soybean trypsin-inhibitor, Proc. Soc. Exper. Biol. & Med. 71: 665–668, 1949.
41. Murray, M. R., and Stout, A. P.: Adult human sympathetic ganglion cells cultivated in vitro, Am. J. Anat. 80: 225–274, 1947.
42. Nantz, F. A., and Blatt, H.: The application of a tissue culture technique in the clinical evaluation of bacterial hypersensitivity, Ann. Allergy 5: 554–557, 1947.
43. Olivo, O. M.; Petralia, S., and Ricamo, R.: L'elettrocardiogramma dell' embrione di pollo nel periodo iniziale dell'attivita contrattile cardiaca, Mem. Accad. Sc. Ist. di Bologna, 1949.
44. Olivo, O. M., and Ricamo, R.: Attivita contrattile coerente di espianti multipli di miocardio embrionale coltivati "in vitro," Mem. Accad. Sc. Ist. di Bologna, 1950.
45. Ormsbee, R. A.; Cornman, I., and Berger, R. E.: Effect of podophyllin on tumor cells in tissue culture, Proc. Soc. Exper. Biol. & Med. 66: 586–590, 1947.
46. Paff, G. H.: A micro-method for digitalis assay, J. Pharmacol. & Exper. Therap. 69: 311–315, 1940.
47. Paff, G. H.: Detoxification of digitalis in the embryonic chick heart, J. Pharmacol. & Exper. Therap. 70: 235–238, 1940.
48. Paff, G. H., and Johnson, B. B.: Reversibility of digitalis action, Proc. Soc. Exper. Biol. & Med. 44: 155–159, 1940.
49. Paff, G. H., and Johnson, J. R.: The behavior of the embryonic heart in solutions of ouabain, Am. J. Physiol. 122: 753–758, 1938.
50. Painter, J. T.; Pomerat, C. M., and Ezell, D.: The effect of substances known to influence the activity of the nervous system on fiber outgrowth from living embryonic chick spinal cords, Texas Rep. Biol. & Med. 7: 417–455, 1949.
51. Parker, R. C.: The races that constitute the group of common fibroblasts: III. Differences determined by origin of explant and age of donor, J. Exper. Med. 58: 401–414, 1933.
52. Parker, R. C.: *Methods of Tissue Culture* (2d ed.; New York: Paul B. Hoeber, Inc., 1950).
53. Peterson, E. R. Production of myelin sheaths in vitro by embryonic spinal ganglion cells, Anat. Rec. 106: 66, 1950.
54. Pogogeff, I. A., and Murray, M. R.: Form and behavior of adult mammalian skeletal muscle in vitro, Anat. Rec. 95: 321–336, 1946.
55. Pogogeff, I. A., and Murray, M. R.: Effects of ryanodine on skeletal muscle cells in vitro, Anat. Rec. 103: 79–80, 1949.
56. Pomerat, C. M., and Anigstein, L.: Reticulo-endothelial immune serum (R.E.I.S.): I. Its action on spleen in vitro, Texas Rep. Biol. & Med. 3: 122–141, 1945.
57. Pomerat, C. M.; Drager, G. A., and Painter, J. T.: Effect of some barbiturates on tissues in vitro, Proc. Soc. Exper. Biol. & Med. 63: 322–325, 1946.

58. Pomerat, C. M., *et al.:* Tissue cultures of adult human cerebral cortex, Texas Rep. Biol. & Med. 8: 108–110, 1950.

59. Pomerat, C. M.: Jacobson, W., and Orr, M. F.: A macrophage-promoting factor (MPF) in the blood of rabbits, Am. J. Anat. 84: 1–26, 1949.

60. Pomerat, C. M.; Nowinski, W. W., and Rose, G. G.: Tissue cultures of cells from body fluids: A preliminary report on methods, Texas Rep. Biol. & Med. 8: 521–532, 1950.

61. Porter, K. R.; Claude, A., and Fullam, E. F.: A study of tissue culture cells by electron microscopy: Methods and preliminary observations, J. Exper. Med. 81: 233–246, 1945.

62. Porter, K. R., and Thompson, H. P.: Some morphological features of cultured rat sarcoma cells as revealed by the electron microscope, Cancer Research 7: 431–438, 1947.

63. Porter, K. R., and Thompson, H. P.: A particulate body associated with epithelial cells cultured from mammary carcinomas of mice of a milk-factor strain, J. Exper. Med. 88: 15–24, 1948.

64. Rioch, D.: The morphology and behavior of the migratory cells in tissue cultures of the chick's spleen, Anat. Rec. 25: 41–57, 1923.

65. Rose, J. M.; Pomerat, C. M., and Danes, B.: Tissue culture studies of ciliated nasal mucosa in man, Anat. Rec. 104: 409–420, 1949.

66. Sanford, K. K., *et al.:* The measurement of cell proliferation in tissue culture by enumeration of nuclei, Anat. Rec. 106: 243, 1950.

67. Shelton, E.: On the length of time required to establish a stable morphologic pattern in cultured cells, Anat. Rec. 106: 246, 1950.

68. Slabeycius, M., and Fischer, A.: An improved technique for morphological studies of tissue cells in plasma media, Acta Anat. 9: 420–422, 1950.

69. Ulloa-Gregori, O., *et al.:* The effect of ascitic fluid and of blood serum on the outgrowth of epidermal cells from the human skin in tissue culture, Texas Rep. Biol. & Med. 8: 400–409, 1950.

70. White, P. R.: Prolonged survival of excised animal tissues in vitro in nutrients of known constitution, J. Cell. & Comp. Physiol. 34: 221–236, 1949.

71. Willmer, E. N.: Carbohydrate metabolism of chick fibroblasts in vitro, J. Exper. Biol. 18: 237–256, 1942.

CELL-PARASITE RELATIONS IN TISSUE CULTURE

Cultivation of Intracellular Protozoa

HERTHA MEYER, *University of Brazil*

For the cultivation of intracellular protozoa it is first necessary to have good growing cells, and all the tissue culture techniques described by Parker (15) and Cameron (1) are suitable for this purpose. If the particular investigation concerns the morphology of the parasites or the infested cell, the cover slip method is best, but if the research is of a more physiologic nature, methods which provide more cells, e.g., cultivation in Carrel flasks, roller tubes or on perforated cellophane are preferable.

If a culture in artificial medium is available, as in the case of *Trypanosoma cruzi* and *Leishmania donovani*, a drop of this culture liquid put on the surface of the tissue explant can be used to infect the cells (6, 16). When no such culture exists, as in toxoplasma and the exoerythrocytic forms of the various plasmodium strains, cultivation can be begun with tissue fragments from the infected animal (3, 4, 8, 13, 17); the blood of animals containing parasites can also be used to infect normal tissue cultures (9, 16).

It has been observed that the host cells are destroyed by the parasites and that 1 parasite gives origin to many new ones during its intracellular life. Thus, ultimately the tissue culture growth is depressed and finally arrested, so that the new parasites can no longer find cells to infest. Organisms like toxoplasma and the exoerythrocytic forms of *Plasmodium gallinaceum* cannot survive outside cells, so if the protozoan strain is to be maintained in tissue cultures, fresh tissue must be added to the infected culture. At the next transfer a fragment of embryonic or other tissue, or an uninfected tissue culture, is therefore placed in contact with the infected material and is invaded by the parasites during the next 2 days of cultivation. Various tissues can be used, as every vigorous cell, normal or malignant, serves as a host for the parasites (10). Strains of *T. cruzi* and of toxoplasma have been maintained in this manner for 4 and 3 yr, respectively, without loss of their special characteristics or their virulence (12, 14). Interesting changes, however, have been observed in *P. gallinaceum*, which developed a sort of tropism for the reticuloendothelial tissue during cultivation over only a few months (8, 9).

As already stated, for morphologic study of the host cell or the parasite, cover slip techniques are recommended, and the explants are put directly on thin histologic cover slips. Use of double cover slips, as in the Maximow method, is not advisable when observation of living parasitized cells with high magnification is required. It is unnecessary to transfer the cover slip cultures 3 times a week; instead they can be washed with Tyrode solution and diluted embryo extract and maintained without transfer for at least 1 week. The washing is done by putting 1 drop of Tyrode solution on the culture surface for a few minutes, then replacing it by 1 drop of diluted extract, which is removed after a few minutes. With this simple modification of the original cover slip technique, observation of a single cell need not be interrupted before the cycle of a parasite is completed. Cultures treated in this manner flatten very much, so that often even the center of the explant can be observed as easily as the periphery of the culture. The use of diluted plasma (1:2 or 1:3) helps the culture to spread and gives much better coloration when it is later fixed and stained in toto.

Fixation in Bouin's, Zenker's or Regaud's fluid gives satisfactory results. Fixation must not last more than 5 min; the culture should 1st be washed in saline solution to remove the remaining extract from the surface, for otherwise the preparation will appear dirty when stained. Hematoxylin Ehrlich, iron-hematoxylin and Giemsa stains, the latter especially for plasmodium cultures, give good images of both cell and parasites.

The life of the intracellular protozoa is closely related to that of the cells, and as long as the host cells are in good condition the parasites also proliferate. When first infested the cells apparently are not affected by the parasites, but continue their normal activities, migrating, pulsating and even multiplying. In all instances observed, however, the parasites eventually destroy the cells by their growing masses. *Trypanosoma cruzi* then transform from the leishmania into the flagellated form and leave the destroyed cell largely by their own activities. Toxoplasma and *P. gallinaceum* are released more passively by rupture of the host cells, and both move for a short time only after liberation. Toxoplasma actively penetrates the new cells, but in cultures infected with *P. gallinaceum* only phagocytosis of the parasites by the neighboring cells has been observed. These 2 forms are obligatory intracellular parasites and soon degenerate if they do not find another host or are not phagocyted by new cells. It is important therefore to add fresh tissues to the infected cultures in time to prevent loss of a strain.

Inside the cells the parasites seem to be well protected and, so long as the host cells survive, they are unaffected by various abnormal conditions such as low temperatures and pH alterations. After incubation for 24 hr, tissue cultures can be maintained at room temperature for some weeks without transfer (2). The cells remain in a state of very slow proliferation, but resume normal growth as soon as they are transferred to fresh medium and incubated again. Parasitized cells also can be kept at room temperature for about 25 days (11). The parasites survive as long as the host cells remain alive and, like the cells, continue to proliferate when they are transplanted and returned to the incubator. It is very useful always to maintain some cultures at room temperature as a reserve in order to preserve the strain and also to permit interruption of the routine.

All the methods of investigation used for normal and malignant cells in tissue cultures can be applied also to infected cells. Drugs can be tested on the parasitized tissue cultures (7, 18), and infected cultures are easily injected into animals.

Protozoa which have been cultivated successfully in tissue cultures are *T. cruzi*, *L. donovani*, toxoplasma and the exoerythrocytic forms of *P. gallinaceum*, *P. relictum* (5) and *P. lophurae* (19).

REFERENCES

1. Cameron, G.: *Tissue Culture Technique* (New York: Academic Press, Inc., 1950).
2. Fischer, A.: Arch. f. exper. Zellforsch. 2: 303, 1925.
3. Guimaraes, F. N., and Meyer, H.: Rev. brasil. de biol. 2: 123-129, 1942.
4. Hawking, F.: Tr. Roy. Soc. Trop. Med. & Hyg. 39: 245-263, 1946.
5. Hawking, F.: Tr. Roy. Soc. Trop. Med. & Hyg. 40: 183-188, 1946.
6. Hawking, F.: Tr. Roy. Soc. Trop. Med. & Hyg. 41: 4, 1948.
7. Kofoid, C. A.; Wood, F. D., and McNeil, E.: Univ. California Pub. Zool. 41: 3, 1935.
8. Lewert, R. M.: Science 107: 775, 1948.
9. Meyer, H.: Rev. brasil. de biol. 9: 211–216, 1949.
10. Meyer, H., and Chagas, C.: An. Acad. brasil. cienc., 1950.
11. Meyer, H., and Xavier de Oliveira, M.: Rev. brasil. de biol. 3: 3, 1943.
12. Meyer, H., and Xavier de Oliveira, M.: Rev. brasil. de biol. 5: 145–146, 1945.
13. Meyer, H., and Xavier de Oliveira, M.: Rev. brasil. de biol. 7: 327–333, 1947.
14. Meyer, H., and Xavier de Oliveira, M.: Parasitology 39: 1, 2, 1948.
15. Parker, R. C.: *Methods of Tissue Culture* (2d ed.; New York: Paul B. Hoeber, Inc. 1950).
16. Romana, C., and Meyer, H.: Mem. Inst. Oswaldo Cruz, Vol. 37, no. 1, 1942.
17. Sabin, A. B., and Olitzki, P. K.: Science 85: 336–338, 1937.
18. Tonkin, I. M.: Brit. J. Pharmacol. 1: 163–173, 1946.
19. Tonkin, I. M., and Hawking, F.: Tr. Roy. Soc. Trop. Med. & Hyg. 41: 407–414, 1947.

APPARATUS FOR EXAMINING CELLS CULTIVATED IN VITRO

Phase Microscopy for Tissue Culture

OSCAR W. RICHARDS, *American Optical Company*

LIVING cells of tissue cultures and other nearly transparent specimens may be observed with the phase microscope. By means of a diffraction disk in the objective and an annular diaphragm in the condenser, a bright field microscope becomes a phase microscope. Small differences in refractive index and size of mitochondria, chromosomes and other cellular constituents affect light passing through them by an alteration of the phase relations of the different light waves, and slight amounts of pigment or absorption also may alter the amplitude of the light waves. These changes are converted by the phase microscope into visible detail. Thus in phase microscopy the contrast of nearly transparent specimens containing detail from differences in optical path (*refractive index* × *thickness*) or from slight amounts of absorption may be increased or decreased; or reversed and increased or decreased.

Phase changes were first used in microscopy near the end of the 19th century. Zernike's (1932) formulation, the development of semitransparent and antireflecting coatings permitting commercial construction and the generalization of the theory by Bennett *et al.* (1) led to a phenomenally rapid development and application of the method. Details on the history and theory and a bibliography are given in Bennett *et al.* (2). All the early writers pointed out the advantage of the new method for study of tissue culture, but early application was limited until methods were developed to permit utilization of Carrel flasks and other growth chambers in common use (12–14).

APPARATUS

While the diffraction disk may contain optical path differences arranged in any form, an annulus is symmetrical with less directional effects and has generally superseded single lines or a cross of lines. An annulus (Fig. 1), or circular window, is placed in the focal plane of the condenser and light *L* passing through it is fo-

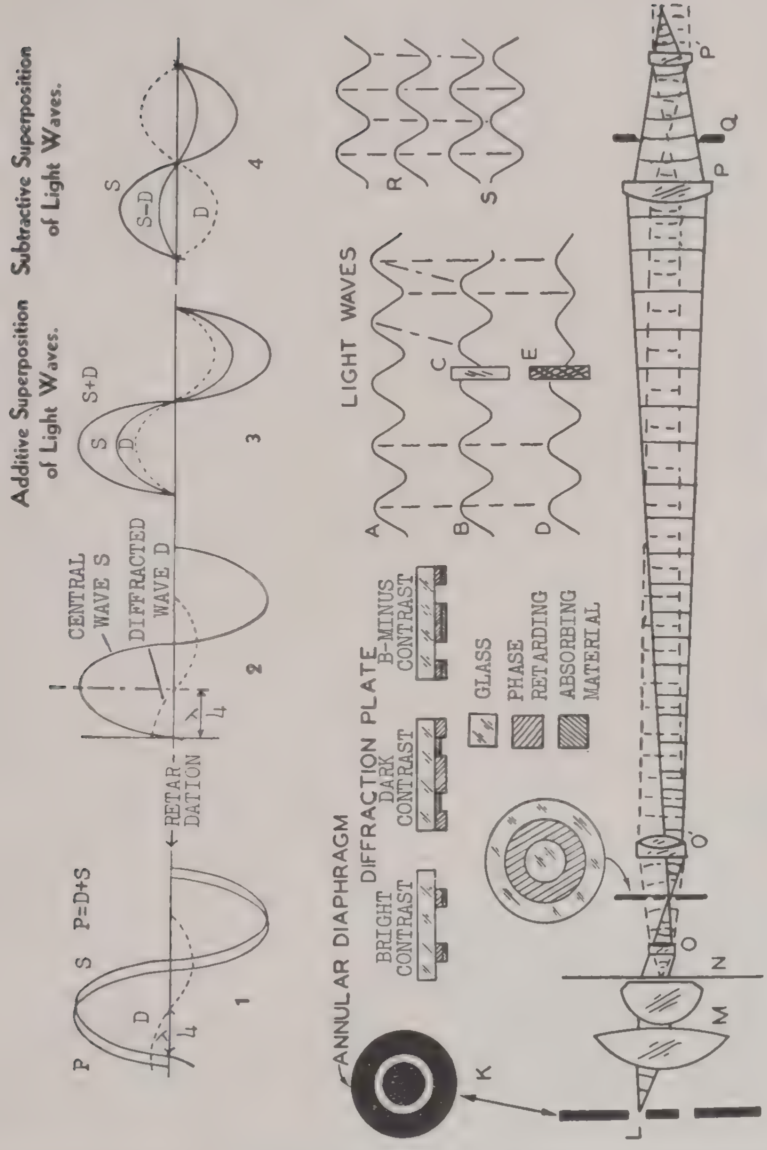
Additive Superposition of Light Waves.
Subtractive Superposition of Light Waves.
P
S
P=D+S
D
λ/4
RETARDATION
CENTRAL WAVE S
DIFFRACTED WAVE D
λ/4
1
2
S
D
S+D
3
S
S−D
D
4
ANNULAR DIAPHRAGM
K
DIFFRACTION PLATE
BRIGHT CONTRAST
DARK CONTRAST
B-MINUS CONTRAST
GLASS
PHASE RETARDING
ABSORBING MATERIAL
LIGHT WAVES
A
B
C
D
E
R
S
L
M
N
O
O'
P
Q
P'

FIG. 1.—Phase microscopy for tissue culture.

cused to give a bright annular image at the back focal plane of the objective. The diffraction plate is a piece of optical grade glass containing a ring of absorbing or retarding material, or both, of such size that it just covers the image of light from the annulus in the condenser. When a specimen is placed on the stage it deviates much of the light through the rest of the area of the diffraction plate. Thus the diffraction plate makes possible selectivity, changing the direct light from the background and the deviated light from the specimen so that the visible contrast is altered for best visibility.

A light wave *B* passing through a clear region of greater refractive index *C* is retarded, or slowed, in comparison with a wave *A*, but such out of phase difference is neither visible nor photographable. When a ray *D* passes through an absorbing medium *E* it is unchanged in phase but reduced in amplitude, and such a change is visible. Two rays of the same phase *R* and amplitude combine in the image, giving greater brightness, but when of opposite phase *S* the combination cancels and no light is seen.

In *1* is seen a ray from a particle and in *2*, one from its surround. *3* and *4* show the additive case and the subtractive case with increased and decreased brightness. The diffraction plate can consist of 4 possible arrangements of the absorbing and retarding materials, 3 of which are useful in applied microscopy. A region of the specimen having greater optical path appears lighter than its surround when both the retarding and the absorbing materials are placed to control the direct (background) light by an A+ plate. Such a region appears dark with an A− plate when the absorbing medium controls the direct light and the retarding medium affects the deviated (specimen) light, or with the B− plate with both media affecting the deviated light.

Change of the amount of absorption or retardation, or both, permits change of the visual or photographic contrast of the specimen. Variable phase systems have been devised, although the experimental models have not proved satisfactory for commercial production. Some manufacturers have followed the lead of Zeiss with a single dark (A− type) contrast; Spencer and others have found a variety of contrasts and types of diffraction plates necessary to meet the demands of the microscopists. The annular diaphragm may be variable and combined with a panchromatic illuminating unit, or several diaphragms may be used singly or mounted in a revolving turret. The size of the condenser diaphragm depends on the numerical aperture of the objective used and on the geometry of the diffraction plate. For a more complete description of equipment and conditions governing contrast, see Bennett *et al.* (2).

The standard equipment is useful with regular slides and cover glasses of 0.18 mm thickness. For thicker preparations, some change in the condenser is required. For slides not over 4.5 mm thick a corresponding part of the upper lens of the microscope condenser may be removed. For thicker preparations, long working distance condensers are required so that the image of the annulus can be focused onto the diffraction plate. For these the manufacturers' catalogs and direction sheets should be consulted.

In use, the condenser is centered to the diffraction plate, the annulus added and centered to the diffraction plate so that its image is superimposed on the ring on the diffraction plate. Centering is facilitated by a small telescope used in place of the ocular. When long focus condensers are used with culture flasks the centering is very important, otherwise the contrast may be markedly reduced. If the culture flask is wedge-shaped it may deviate the light from the condenser and make centering impossible unless a prism (such as is used in spectacles) is placed on top of the condenser. With roller tube cultures only those cells at the top of the tube can be seen clearly. Hollow ground, concave depression slides may not be used because they act as a lens and prevent alinement of the equipment. Gey has made cells with straight sides and cover slips on each side.

Bright A+ contrast may reveal detail in cells better than dark contrast and is preferable for small detail. The high contrast is better for small details than the low contrast, whereas the low contrast shows larger cells better. The medium contrast often serves as a useful compromise. Dark B− contrast shows less halo around the details than does the A− dark contrast diffraction plate. The B− is especially good with slightly pigmented cells and supravitally stained cells.

The 16 mm low bright contrast ($0.2A + 0.33\lambda$) is useful for the examination of cultures. With the 4 mm objective the low and medium bright (A+) and dark (A−; B−) have found favor in this field, and these and the light bright contrast ($0.07A + 0.25\lambda$) are used with the oil immersion lens.

Comment.—Perry (11) demonstrated spirochetes of syphilis in a culture of testicular cells. Motion pictures made with the phase microscope have revealed considerably more information (3, 4, 6, 9, 10), and Hughes and Swann (8) combined phase and polarization microscopy for analysis of mitotic phenomena. Other examples are considered elsewhere in this chapter. Photography requires only slightly longer exposures (12). The phase microscope makes possible the observation of detail in living, unstained tissue cultures that otherwise would be seen only after time-consuming staining methods and is making possible the comparison of living and postmortem cytology and histology.

REFERENCES

1. Bennett, A. H., *et al.*: Phase microscopy, Tr. Am. Micr. A. 65: 99–131, 1946.
2. Bennett, A. H., *et al.*: *Phase Microscopy* (New York: John Wiley & Sons, Inc., 1951).

3. Danes, B.: Ciliated pulmonary epithelium of Triturus viridescens in tissue culture, Anat. Rec. 103: 590–591, 1949.
4. Farris, E. J.: A new method of semen analysis, Anat. Rec. 97: 429, 1947.
5. Farris, E. J.: *Human Fertility and Problems of the Male* (White Plains, N. Y.: Author's Press, 1950).
6. Firor, W. M., and Gey, G. O.: The study of malignant cells with phase difference microscopy, Ann. Surg. 125: 604, 1947.
7. Hughes, A. F. W.: The technique of cinephotomicrography of living cells, J. Roy. Micr. Soc. 69: 53–64, 1949.
8. Hughes, A. F. W., and Swann, M. M.: Anaphase movements in the living cell, J. Exper. Biol. 25: 45–70, 1948.
9. Michel, K.: Die Darstellung von Chromosomen mittels die Phasenkontrastverfahrens, Naturw. 29: 61–62, 1941.
10. Michel, K.: Das Phasenkontrastverfahren fur zytologische Untersuchungen. Naturw. 37: 52–57, 1950.
11. Perry, W. L. M.: Cultivation of *Treponema pallida* in tissue culture, J. Path. & Bact. 60: 339–342, 1948.
12. Richards, O. W.: Growth of bacterial and fungal colonies as seen with the phase microscope, J. Bact. 54: 4, 1947.
13. Richards, O. W.: Phase photomicrography, J. Biol. Photog. A. 16: 29–38, 1947.
14. Richards, O. W.: Phase microscopy in bacteriology, Stain Technol. 23: 55–64, 1948.

Electron Microscopy of Cultured Tissue Cells

KEITH R. PORTER, *Rockefeller Institute for Medical Research*

The limited resolving power of visible light and the optical microscope have prevented, until recently, the direct examination of the structure and organization of protoplasm as it exists in dimensions less than 0.2 μ. The presence of submicroscopic particulates could be discerned by means of dark-field microscopy, but little more than their existence could be demonstrated. It is only in the last decade that the electron microscope has extended our range of vision down to these "submicroscopic" levels, and although the information thus far available from this new technique on tissue cells is meager, it is sufficient to indicate that we are on the threshold of a period of rapid progress in the analysis of structural-functional relationships.

Electron microscopy makes one important demand of the specimen: it must be very thin. This arises from the fact that a ray of electrons has a much smaller power of penetration than a ray of light. Thus whole cells as they occur in most animal tissues are much too thick, and sections of 1–2 μ, which might be excellent for light microscopy, appear totally opaque in the electron microscope. One is obliged, therefore, in electron microscopy to study minute fragments of cells, extremely thin sections (not more than 0.2

0.3μ) or cells selected especially for their thinness. Cultured tissue cells fall in the last group.

Specimen preparation must take into account certain additional features unique to electron microscopy. Staining with the aim of differentiating better between structures cannot be achieved with dyes as for light microscopy; it must rather be accomplished by a differential deposition within the material of a heavy element or elements such as compounds containing tungsten, osmium or iodine. Another consideration in specimen preparation is imposed by the vacuum necessary in the microscope during operation. Because of this the object examined is thoroughly dried and must in advance be protected as far as possible against distortions arising from drying, either by chemical fixation or by providing an embedding matrix.

Probably of all the materials available for cytologic studies by these means, none combines as many virtues as cells grown in culture. In the form they assume when growing out on a rigid supporting surface, they are always sufficiently thin at their margins and occasionally at their centers for excellent microscopy. It is not necessary, as with thin sections, to translate the 2-dimensional picture into a 3-dimensional image. Instead, the structure observed is that of a complete cell with parts oriented as in the living unit. Furthermore, their isolation in culture permits the precise control of their environment in experimentation designed to reveal structural-functional relationships, and the growing undifferentiated unit may be examined and compared with its differentiated descendent.

PROCEDURES

The methods (2, 5) involved in the preparation of cells for electron microscopy are in no respect more difficult than those required for light microscopy. They fall into a few separate categories such as preparation of the culture glassware, the culturing, the fixation of the cells and finally their transfer to the electron microscope screens.

a) Culture glassware.—The support for materials to be examined in the electron microscope must, of course, be transparent to electrons and is usually a thin film of celloidin or a resin referred to commercially as Formvar.* Since this film has to be under the tissue cells after they have grown out in culture, it is added as a coating to the glass surface before the cultures are set up. This coating is applied by dipping the cover glass, to be used as a culture support, into a dilute solution of celloidin (0.5% in butyl acetate) or Formvar (0.3% in ethylene dichloride). During the dipping and drying the cover glass should be backed by a heavier glass or metal to supply heat for evaporation of the solvent. If this precaution is neglected, water condenses in the film and spoils it. When dry, the film-coated cover glass is sterilized by dipping it in 70% alcohol made up from the best grade, redistilled alcohol.

---

* Formvar (grade No. 15-95) is the trade name for a polyvinyl formal plastic manufactured by the Shawinigan Products Corp., New York.

b) Culturing.—Almost any type of culture may be employed. Plasma-free cultures are favored because a large part of the cells which grow out are on the surface of the slide and the medium can be simply rinsed away in preparing the cells for fixation.

If a plasma clot is employed a certain sequence of procedures has to be followed to insure that the clot may later be removed without disturbing the layer of cells adherent to the film-coated glass. Thus the explant should be placed on the culture surface first, and the balanced saline solution accompanying it spread around over the area expected to be covered by the cell colony. The excess of this solution is then withdrawn, and before any drying can ensue the plasma-nutrient mixture is added. The film of Tyrode solution added 1st prevents the clot from adhering to the slide over the region adjacent to the explant, and the cells which grow out into this region will later remain attached to the film when the clot is removed.

c) Fixation of cells.—Before fixation, the selected cultures are freed of clot and medium while immersed in balanced saline solution and thereafter rinsed in 2 clean baths of the same (Earle's formula preferred). While still bathed in a drop of this they are inverted over a well slide (Maximow type) containing 0.2 cc of a 2% water solution of osmium tetroxide. The vapors from the OsO_4 instantly fix the cells and faithfully preserve the protoplasmic structures. Fixation may be interrupted at the end of 5 min or continued for 16-20 hr, depending on what components of the cell are being examined. (Formaldehyde is another valuable fixative. It should be used as a neutral 8% solution in physiologic saline and applied directly to the material.) Following fixation the cells, while still attached to the cover glass, are washed in water for 20 min before being transferred to the wire grid used to support the thin film during microscopy.

d) Transfer of cells to microscope screen.—Transference of the cells from the slide on which they have been growing and fixed to the wire mesh disks is accomplished by peeling away a small portion of the film supporting the cells and floating it over the grid. This operation is performed with the aid of a dissecting microscope and admittedly requires practice. In detail, an area or group of cells, which for one reason or another is good for microscopy, is selected and the continuity of the film around it is broken with the point of fine watchmaker's forceps. This leaves the selected cells attached to a small island of film only slightly larger than the 3 mm screen. In the next step this film is peeled away from the glass and under water floated onto the grid. In this position it is secured to the grid with the forceps and lifted with the grid out of the water. It is quickly drained on filter paper and permitted to dry, after which it is ready for microscopy.

Preparations made by these procedures have provided material for observations on cell-fine structure (5, 8), the growth of viruses in cells (1, 3, 7), the differences between normal and malignant cells (4, 6) and the development of extracellular products of tissue cells, such as collagen (9).

REFERENCES

1. Claude, A.; Porter, K. R., and Pickels, E. G.: Electron microscope study of chicken tumor cells, Cancer Research 7: 421, 1947.
2. Martin, A., and Tomlin, S. G.: A technique for the cultivation and preparation of tissue cultures for electron microscopy, Biochim. et biophys. Acta 5: 154, 1950.
3. Murphy, J. S.; Karzon, D. T., and Bang, F. B.: Studies of influenza A (PR8) infected tissue cultures by electron microscopy, Proc. Soc. Exper. Biol. & Med. 73: 596, 1950.
4. Oberling, C., *et al.*: Images des cellules cancéreuse au microscope electronique, Bull. Assoc. franç. p. l'étude du cancer 37: 97, 1950.
5. Porter, K. R.; Claude, A., Fullam, E. F.: A study of tissue culture cells by electron microscopy: Methods and preliminary observations, J. Exper. Med. 81: 233, 1945.
6. Porter, K. R., and Thompson, H. P.: Some morphological features of cultured rat sarcoma cells as revealed by the electron microscope, Cancer Research 7: 431, 1947.
7. Porter, K. R., and Thompson, H. P.: A particulate body associated with epithelial cells cultured from mammary carcinomas of mice of a milk-factor strain, J. Exper. Med. 88: 15, 1948.
8. Porter, K. R.: The fine structure of the cytoplasm of cultured tissue cells, Anat. Rec. 100: 704, 1948.
9. Porter, K. R., and Vanamee, P.: Observations on the formation of connective tissue fibers, Proc. Soc. Exper. Biol. & Med. 71: 513, 1949.

SUBJECT INDEX

[Page numbers printed in bold face indicate original contributions to this volume.]

A

Acetone: extraction for T-1824 in turbid plasma, 42
Adrenal glands (infantile): culture by watch glass technique, 237 f.
Adrenaline: histochemical demonstration, 8
Amino acids
  in bovine serum albumin, 204
  in lactoglobulin, 204
  for tissue culture media, 202 ff.
Anesthesia: for pancreatic surgery, 149
Anticoagulants
  oxalate (Heller-Paul), 63n.
  sodium polyanethol-sulfonate, 75n.
Antipyrine
  N-actyl 4 aminoantipyrine, *see* NAAP
  in plasma—measurement, 33 f.
    drugs interfering with, 33
    reagents for, 33
    standard curve, 34
  renal excretion, 37
  sources, 32n.
  sterilization of, 32n.
  for total body water measurement, 31 ff.
Arginine: histochemical demonstration (Baker reaction), 7
Ascorbic acid: histochemical demonstration, 7
Auerbach's plexus: Klein pouch without, 144
Autoradiography: in radioactive tracer studies in tissue cultures, 249

B

Balloons: for registration of intralumen pressures, 110
Bismuth: histochemical demonstration, 6
Blood, *see also* Plasma
  in antipyrine estimation of total body water—samples for, 32
  for flame photometry, 88, 93
  volume—normal values, 44
Blue
  Evans'
    extinction coefficient in plasma, 41n.
    for plasma volume estimation, 39 ff.
    stability, 41n.
  Prussian, for hemosiderin-iron, 4
Bone
  sodium and its isotope in, 55
  techniques of cultivation, **234** ff.
    hanging drop method, 236 f.
    watch glass method, 235 f.
Buffers: for histochemistry, 25 ff.
  0.2M acetate, 25
  0.2M 2-amino-2-methyl-1,3-propanediol, 26
  0.05M barbital, 26
  0.2M borate, 26
  0.2M citrate, 25
  0.2M maleate, 25
  0.2M phosphate, 25
  0.2M tris(hydroxymethyl)aminomethane-maleate, 26

C

Calcium
  -cobalt demonstration of alkaline phosphatase, 17
  histochemical determination, 3 f.
    carbonate, Kóssa test for, 3
    phosphate, Kóssa test for, uric acid removal, 4
  ion concentration
    biologic method of estimation, **68** ff.
    calculation from total Ca and protein in blood, 78 f.
    difficulties, 78 f.
    evaluation, 77 f.
    frog's heart in, 69
    —preparation—technic, 70
    —sensitivity, 69, 76, 77
    procedure, 69 ff.
    sensitivity, 76 f.
    solutions of reference, 71 ff.
    —preparation, 72 f.
    —variations in, 77
    temperature for, 77
    test technique, 73 ff.

Cancer, *see also* Cells, malignant
  chemotherapy, tissue culture screening of agents, **272** ff.
Cannulas
  for pancreatic fistulas (semipermanent), 154, 155
  for pancreatic juice collection through duodenal fistula, 161, 162
  for stomach pouches, 125
  Straub, modified, 70
  for Thomas pouch, 139
Carmine
  Best's, for glycogen, 10
  mucicarmine (Mayer's) for mucin, 11
Cellophane: perforated, for replicate cell culture, 218
  medium for, 219
Cells, *see also* Tissue Culture
  enterochromaffin — demonstration, 8 f.
    azo-coupling reaction, 9
    reducing power (Ag technique), 8
  malignant
    characteristics, 263
    —electron microscopy of, 263
    culture method, 212 ff.
    sources in medical installations, 211
    in tissue culture screening in experimental cancer chemotherapy, **272** ff.
  microscopy in tissue cultures
    electron, **288** ff.
    phase, **284** ff.
  protozoan cultivation in, 281 ff.
  radioactive tracer studies in tissue cultures, **247** ff.
  tissue
    pure strain, method of development, **217**
    single isolated, method for growth, **217** f.
Chloride
  distribution
    in body cells, 54
    in extracellular fluid, 54
  extracellularity of, 54 f.
  radioactive
    available isotopes, 57
    behavior in body, 54
    dosage, 58 ff.
    —safe, calculations, 59 ff.
    normal extracellular values, 65 f.
p-Chloroaniline-N-phosphonic acid: synthesis for phosphamidase determination, 23
Cholesterol: histochemical demonstration
  digitonine reaction, 13
  Schultz reaction, 13
Cholinesterase: histochemical demonstration, 22 f.
  fixation and embedding, 16
  synthesis of myristoyl choline, 22
Chromaffin, 8
  enterochromaffin cells
    azo-coupling reaction, 9
    reducing power (Ag technique), 8
Chromic acid: for polysaccharide identification, 9 f.
Cobalt-calcium: demonstration of alkaline phosphatase, 18
Copper: histochemical demonstration, 5

D

Detergents: for flame photometry, 95
Deuterium: for total body water estimation, 31
Diabetes mellitus: T-1824 determination in plasma of, 42
Diazonium compounds: for alkaline phosphatase demonstration, 18
Digestive tract: intralumen pressure registration, **109** ff.
  balloon (naked) for—errors, 110
  comparison of methods, 122
  open tube electromanometer technique, 119 ff.
  physical considerations, 110 ff.
  pressure conduction method, 114 ff.
    conducting medium, 114, 115
    connecting tube construction, 116, 117
    connecting tube dynamics, 114, 115
    pressure recorder for, 114
    tip construction, 116
    tip dynamics, 114, 115
  recording device
    dynamic requirements, 111
    volume/pressure coefficient, 110
  2-channel optical manometer method, 116 ff.
    apparatus, 116 f.
    procedure, 118
Digitonine: for cholesterol demonstration, 13
Drugs
  assay by tissue culture, 260 ff.
    culture methods, **261** f.
    end-points in evaluation, **262**, **270**, **271** f.
    flask method (Parker), **271**
    Medawar's hanging drop technique, **271**
    primary explants vs. cell strains, **261**

Drugs (*cont.*)
        from primary outgrowth on cover slips, **266** f.
    interfering
        with antipyrine estimation, 33
        with NAAP estimations, 36
Duodenum: fistula for collection of pancreatic juice, 160, 161 ff.
Dyes: for plasma volume estimation, **38** ff.
    T-1824 procedure, 39 ff.

E

Edema
    antipyrine estimation of total body water with, 32
    radioisotope sample collection in, 63
    thiocyanate space determination in, 49, 50, 52
Electromanometer (Sanborn): for intralumen pressure registration, 119
Endocrine glands (infantile): tissue culture techniques
    in anterior eye chamber, **240**
    human tissues, on watch glass, **241**
    on watch glass, in moist chamber, **237** ff.
Enterogastrone: in gastric secretory inhibitor assays, 176
    response to histamine, 181
Enzymes
    from gastric mucosa—localization, 193
    hydrolytic—histochemical demonstration, 16 ff.
        embedding of tissue for, 16
        fixation, 16
        paraffin embedding, 16
    and mitochondria, in study of drug action on living cells, 264
    oxidative—histochemical demonstration, 14 f.
Esophageal fistulas, 123 ff.
    animal feeding with, 127
    complications, 128
    procedure, 126 ff.
Esterase: histochemical demonstration, 20 ff.
    differentiation from lipase, 19
    fixation and embedding, 16
    naphthol ester technique, 21
    tween techniques, 20
Eye: anterior chamber, for tissue culture, **240** f.

F

Feces: preparation for flame photometry, 88, 93
    dry ashing, 93
    nitric acid extraction, 94
Fistulas
    duodenal, for pancreatic juice collection, 160 ff.
    esophageal, operation for, 126 ff.
    gastric, for gastric secretory inhibitor assays, 174
    pancreatic, 154 ff.
        care of animals with, 158 f.
        permanent, 156 ff.
        retrograde external (McCaughan), 158, 159
        semipermanent, 154 ff.
Fluid, extracellular: Na and Cl in, 54
Fluid, extracellular: volume determination
    with radioisotopes, **53** ff.
        calculations, 65 f.
        critique, 65 ff.
        dose to be administered, 58 ff.
        —safe, calculations for, 59 ff.
        evaluation, 53 ff.
        isotope of choice, 57
        normal values, 65, 66
        procedure, 62 ff.
        sample preparation, 62 ff.
        —for counting, 63
        —for injection, 62
    with thiocyanate, **48** ff.
Food: preparation for flame photometry, 88, 93
    dry ashing, 93
    nitric acid extraction, 94
Frog heart: in Ca^{++} determination, 69
Fuchsin sulfuric acid, *see* Reagents, Schiff's

G

Gastric juice
    collection from total stomach pouches, 177
    radiosodium excretion from, 55
Glycogen, 9
    histochemical demonstration, 10
        Best's carmine stain for, 10
Gold: histochemical demonstration (Elftman method), 6
Gonads (infantile): watch glass tissue culture techniques, 238
    human fetal tissue, 244 f.

H

Haemo-Sol: for flame photometry, 95
Hemoglobin, 4
    in Lison's Zn-leuco technique for peroxidase, 15
    staining technique, 5
Histamine: for assay of gastric secretory inhibitors, 175, 176

Histamine (*cont.*)
  dosage for, 180
  enterogastrone response, 181
  procedure, 177 ff.

I

Insulin-acidity test of vagal integrity, **166** ff.
  conditions for validity, 168
  equivocal result, 170
  interpretation, 170
  negative result, 170, 171
  positive result, 170, 171
  procedure, 168 ff.
Iron
  hemosiderin, Prussian blue localization method, 4
  masked, 4
    localization technique, 5

K

Kidneys: antipyrine excretion, 37

L

Lactoglobulin: amino acid composition, 204
Lauroyl: preparation for cholinesterase demonstration, 22
Lead: histochemical demonstration, 6
Leishmania donovani: intracellular cultivation, 281 ff.
Lipase: histochemical demonstration, 20 f.
  differentiation from esterase, 21
  fixation and embedding, 16
  tween techniques, 20
Lipids: histochemical demonstration, 12 ff.
  stains for, 13
Liver: cirrhotic, Cu demonstration in, 5

M

Manometers: for intralumen pressure registration
  optical, 2-channel open tube, 114 ff.
    application, 116 ff.
  U-tube, 111
    inadequacies, 112
Media: for tissue culture, 201 ff.
  antiobiotics for contamination, 268
    in perfusion chamber, 275
  for cellophane replicate cultures, 219
  commercial, 209 f.
    NNP assay, 225 ff.
  for double cover slip technique, 214
  embryo juice, dialyzed, 201
  synthetic, 201 ff.
    amino acid supplements, 202 ff.
    analytic and supplementation methods, **201** ff.
    Fischer's dialyzed, 201 ff.
    for plant tissues, 206
    protein supplements, 204
    solution V-614, 204
    true, defined media, **206** f.
Mercury: histochemical demonstration, 6
Microscopy, for tissue cultures
  electron, **288** ff.
    of malignant cells, 263
    tissue preparations, 288, 290
  phase, **284** ff.
    apparatus, 284 ff.
    applications, 287
    perfusion chamber for, **275** ff.
    tissue preparations, 286
Mucin, 9
  histochemical demonstration, 10
    metachromatic staining, 11
    mucicarmine (Mayer's) stain for, 11
Muscle: skeletal, maintenance in tissue cultures, 215, 269
Myristoyl choline: synthesis for cholinesterase demonstration, 22

N

NAAP
  in plasma—estimation, 36 f.
    drugs interfering with, 36
    reagents, 36
    standards, 37
  sterilization of, 32n.
  sources, 32n.
  for total body water measurement, 34 ff.
    urinary excretion procedure, 34, 35 f.
  in urine—estimation, 36 f.
    reagents, 36
    standards, 37
Naphthol
  for alkaline phosphatase demonstration, 18
  alpha—sublimation technique, 36
  esters, for esterase localization, 21
Nephrotic syndrome
  calculation of blood and red cell values in—errors, 44
  T-1824 determination in plasma of, 42
Nervous system: tissue culture
  ganglia for, 269
  spinal cord explants for, 268
Nomogram: for Ca^{++} determination from total protein and plasma in blood, 77, 78

Nucleic acid
  in determination of cell growth in tissue cultures, 225 ff.
  histochemical demonstration, 12
    desoxyribose, 12
    ribose, 12
  pentose and desoxypentose—estimation in roller tube tissue cultures, 227 ff.
Nylon: for tissue culture vessels in study of radiation effects, 256

O

Ovaries (infantile): watch glass culture techniques, 238
  human fetal tissue, 244 f.
Oxidase: histochemical demonstration
  dopa, 15
  Winkler-Schultze method, modified, 14

P

Palmitoyl
  preparation for cholinesterase demonstration, 22
Pancreas
  ducts
    in dogs, 151
    ligation or avulsion for study of insufficiency, 163
    in rabbit and cat, 152
  excretory function (external), methods for study, 149 ff.
    abdominal incisions, 150 f.
    anesthesia for surgery, 149
    animals for, 149
    duct exposure and cannulation, 151 ff.
    surgical approach, 149 ff.
  fistulas
    care of animals with, 158 f.
    permanent, 156 ff.
    retrograde external (McCaughan), 158, 159
    semipermanent, 154 ff.
  insufficiency, experimental technique, 163
  islets, Zn demonstration in, 5
  juice collection through duodenal fistula, 160 ff.
Paraffin: for hydrolytic enzyme demonstration, 16
Parathyroid glands: human infantile, culture by watch glass technique, 241 ff.
Periodic acid: for polysaccharide identification, 9 f.
Peroxidase: histochemical demonstration (Lison's Zn-leuco technique), 15
Phosphamidase: histochemical demonstration, 23 f.
  fixation and embedding, 16
  synthesis of p-chloroaniline-N-phosphonic acid for, 23
Phosphatase
  acid—histochemical demonstration, 19
    fixation and embedding, 16
  alkaline—histochemical demonstration, 17
    Ca-cobalt method, 17
    fixation and embedding, 16
    naphthol method, 17, 18 f.
Phosphorus: nucleoprotein, estimation in roller tube tissue cultures, 226 f.
Photometer, flame: 79 ff.
  accuracy, 90
  atomizer, 84 f.
    atomizing chamber, 85
  direct intensity, instrument, 81 f.
  filters
    light, 86
    neutral density, 92
  flame, 86
  interferences, 87 f.
  internal standard
    circuits, 83
    instrument, 82 f.
    precision, 90
  location, 87, 92
  photocells, 87
  scope, 89
  selection of instrument, 90
  technical supervision, 89
  variable factors, 84 ff.
Photometry, flame: procedures with, 79 ff.
  calibration curves
    potassium—direct intensity, 98
    readings, 92, 95 f.
    sodium—direct intensity, 92
    —construction, 98
  direct intensity
    determination of Na and K in 1 instrument, 98
    procedure, 98 ff.
  evaluation, 80 ff.
  internal standard
    determination of Na and K with 1 instrument, 102 f.
    procedure, 100 ff.
  potassium
    in serum—readings for, 96
    —values (normal), 103 f.
  readings, 92
    method, 95 f.
  reagents
    "artificial serum," 96 f.

Photometry (*cont.*)
    lithium sulfate, 97, 98
    potassium, 97
    sodium chloride, 97
  samples
    blood dilution, 88, 93 f.
    feces preparation, 88
    —dry ashing, 93
    —nitric acid extraction, 94
    food preparation, 88, 93 f.
    method of introduction, 84
    serum dilution, 88, 93 f.
    tissue preparation, 88, 93 f.
    urine dilution, 88, 93 f.
  serum analysis, 96
    values for Na and K, 103 f.
  solutions—preparation, 93, 96 ff.
  techniques, 91 ff.
Pineal body (infantile): culture by watch glass technique, 240
Pinocytosis, 264
Pituitary gland (infantile): anterior lobe, culture
  in anterior eye chamber, 240 f.
  on watch glasses, 238 f.
Plasma, *see also* Blood
  in antipyrine estimation of total body water, 32 f.
  antipyrine in—measurement, 33
    reagents for, 33
    standard curve, 34
  in NAAP estimation of total body water, 34, 35
  NAAP in—estimation, 36 f.
    drugs interfering with, 36
    reagents, 36
    standards, 37
  thiocyanate in—determination, 51
  turbid—acetone extraction for T-1824 in, 42
  volume—normal values, 44 f.
  volume—dye dilution method, 38 ff.
  volume—dye injection method
    blood sampling, 40
    calculations, 43 ff.
    —of plasma volume, 43
    —of red cell volume, 44
    —of whole blood volume, 44
    corrections
    —for hemolysis, 43
    —for residual dye, 43
    dye concentration determinations
    —in clear samples, 41
    —in turbid samples, 42
    dye injection techniques, 39 f.
    evaluation, 45 ff.
    reproducibility of results, 45
Plasmal, 12
  histochemical demonstration, 14
Plasmalogen, 14
Plasmodia: intracellular cultivation, 281 ff.
Polysaccharides: chemical identification, 9 ff.
  chromic and periodic acid for oxidation, 9 f.
  Schiff's reagent, 9
Potassium
  flame photometer determinations, 79 ff.
  in serum, normal values, 103 f.
Probes: glass, for pancreatic juice collection through duodenal fistula, 161, 162
Protein solvent (Haemo-Sol), 95
Protozoa: intracellular, cultivation techniques, 281 ff.
Pyrexin: in assay of gastric secretory inhibitors, 182
Pyrogens: in assay of gastric secretory inhibitors, 182

R

Radiations, effects on tissue cultures
  methods of study, 255 ff.
  radioactive tracer studies, 247 ff.
Radioisotopes
  for extracellular fluid studies, 48 ff.
    selection of isotope, 57
  precautions, 62, 254
  in tissue culture studies, 247 ff.
    autoradiography, 249
    dosage rate—calculations, 254
    geometric standards, 248
    permeability rate—estimation, 250
    radiation effects, 254
    radiation hazards, 254
Reagents
  for antipyrine estimation in plasma, 33
  for arginine demonstration, 7
  "artificial serum," for flame photometry, 96 f.
  Dische's diphenylamine, 227
  ferric nitrate, 51
  for flame photometry, 96 ff.
    "artificial serum," 96 f.
    lithium sulfate, 97, 98
    potassium, 97
    sodium chloride, 97
  Mejbaum's, modified, 227
  for NAAP estimation in plasma and urine, 36
  for NNP estimation in roller tube tissue cultures, 226
  for pentose and desoxypentose estimations in tissue cultures, 227 ff.

Reagents (*cont.*)
  Schiff's, 10
  for thiocyanate determinations, 51
  zinc, for antipyrine determinations, 33, 36
Reticuloendothelial system: role in plasma volume determinations, 46
Ribonuclease: preparation for staining of ribose nucleic acids, 12
Roentgens: definitions, 59
  equivalent physical, 60

S

Serum: "artificial," for flame photometry, 96 f.
Silver: histochemical demonstration, 6
Silver-methenamine: preparation, 8
Sodium
  in bone, 55
  distribution
    in body cells, 54
    in extracellular fluid, 54
  extracellularity of, 54 f.
  flame photometer determinations, 79 ff.
    calibration curve (direct intensity), 92, 98
  polyanethol-sulfonate, 75n.
  radioactive
    available isotopes, 57
    behavior in body, 54
    in bone, 55
    dosage, 58 ff.
    —safe, calculations, 59 ff.
    excretion time, 55, 56
    normal extracellular values, 65 f.
  in serum, normal values, 103 f.
Stains
  carmine (Best's) for glycogen, 10
  for lipids, 13
  metachromatic, for mucin, 11
  mucicarmine (Mayer's) for mucin, 11
  Prussian blue, for hemosiderin iron, 4
Sterox: in flame photometry, 95
Stomach
  fistulas—for secretory inhibitor assays, 174
  innervation of, 129 f.
  juice
    collection from total stomach pouches, 177
    radiosodium excretion from, 55
  mucosa
    with intact blood supply, 186
    between 2 chambers, apparatus, 187 ff.
  secretion, in vitro study methods, 185 ff.
    enzyme localization, 193 ff.
    from excised stomach sacs—methods, 190 ff.
    mucosa between 2 chambers—apparatus, 187 ff.
    from mucosa with intact blood supply, 186
  secretory inhibitors, assay methods, 173 ff.
    animals for tests, 174
    continuous test, 175
    enterogastrone in, 182
    histamine test, 175, 176, 177 ff., 180
    pyrogens in, 182
    stimuli for test, 175
    2-dose test, 175
Stomach pouches, 123 ff.
  for acute experiments, 128 ff.
    drainage, 129
  Brunschwig subtotal, technique, 137
  cannulas for, 125
  Cope fundus, procedure, 135 ff.
  denervated vagally, 141 ff.
    completely denervated, 145
  Dragstedt procedure, 130 ff.
  everted fundus, Friedman technique, 145
  fistulas for, 125
    for retention pouches, 126
  Frément-Ivy, 141
  Goldberg-Mann, technique, 143
  Heidenhain, technique, 141 ff.
    fistula for, 125
  Hollander fundus, procedure, 133 ff.
  innervated, 129 ff.
    choice of pouch, 129
  Klein, without Auerbach's plexus, technique, 144
  from lesser curvature, technique, 146
  Necheles fundus, procedure, 134 f.
  Pavlov fundus, procedure, 131 ff.
    mucosal area incorporated in, 125
  postoperative care, 146 ff.
  retention, fistula for, 126
    for secretory inhibitor assays, 174
  subdivision by rubber stopper, 128
  Thomas fundus, technique, 138 ff.
    cannula for, 139
  total
    gastric juice collection from, 177
    innervated, procedure, 130 ff.
    for secretory inhibitor assays, 174

Stomach pouches (*cont.*)
  transplanted (Ivy-Farrell), technique, 143

T

T-1824
  extinction coefficient in plasma, 41n.
  for plasma volume estimation, 39 ff.
  stability, 41n.
Thiocyanate space: determination, **48** ff.
  evaluation, 49 ff.
  procedure, 51 ff.
    determinations
    —of space, 52
    —of thiocyanate in plasma, 51
  solutions, 51
Tissue culture
  of adrenal glands, 237 f.
  antibiotics for contaminated tissues, 268
    in perfusion chamber studies, 275
  of bone—techniques, **234** ff.
    hanging drop method, 236 f.
    watch glass method, 235 f.
  in cancer chemotherapy experiments, screening method, **272** ff.
  cellophane (perforated) for, **218**
    medium for, 219
  chemical agents—effects on cells, 260 ff.
    culture methods for, **261**
    end-points in evaluation, **262** ff., 270
    perfusion chamber for, **275** ff.
    primary explants vs. cell strains, **261**
  double cover slip technique, **212** ff.
    explantation mixtures for, 214
    procedure, 213 ff.
    supplies, 212
  drug toxicity studies
    end-points in evaluation, **262**, 270
    —toxic or lethal, titration of, 271 f.
    flask method (Parker), **271**
    Medawar's hanging drop assay, **271**
    methods of culture, **261** f.
    primary explants vs. cell strains, **261**
    from primary outgrowth on cover slips, **266** ff.
  electron microscopy for, **288** ff.
    tissue preparations, 288, 290
  of endocrine glands, 237 ff.
  of gonads, 238, 244 f.
  growth measurement
    by enumeration of nuclei, **219** ff.
    by nucleic acid determinations, **225** ff.
  growth (organized)—in vitro studies, 232 ff.
  media, *see* Media
  in medical installations, **211** ff.
    double cover slip technique, 212 ff.
    material—sources and handling, 211
  NNP estimation—technique, 226
  nucleic acid determinations, **225** ff.
    Dackermann-Patterson method, 230
    Davidson - Leslie - Waymouth method, 227
    Davidson-Waymouth (NNP) method, 226
    Hull-Kirk method, 229
    Steele - Sfortunato - Ottolenghi method, 229
  of ovaries, 238, 244 f.
  of parathyroid glands, 241 ff.
  pentose and desoxypentose nucleic acid estimations—technique, 227 ff.
  perfusion chamber technique, **275** ff.
  phase microscopy for, **284** ff.
    tissue preparations, 286
  of pineal body, 240
  of pituitary (anterior), 238, 240 f.
  proliferation measured by enumeration of cell nuclei, **219** ff.
  protozoa, intracellular cultivation, **281** ff.
  pure cell strains, development of, **217**
  quantitative methods, **217** ff.
  radiation effects, **255** ff.
    culture vessels for study of, 256
    dosage determinations, 257
    of radioisotopes, 254
  radioactive tracer studies, **247** ff.
    applications, 247 f., 252
    autoradiography, 249
    geometric standards, 248
    permeability rate—estimation, 250
  replicate
    cellophane (perforated) for, **218**
    enumeration of cell nuclei, **219** ff.
    preparation and handling, **221** ff.
  roller tube
    NNP estimation—technique, 226
    RNAP and DNAP estimations, 227 ff.
  single cell growth, method, **217** f.

Tissue Culture (*cont.*)
  spinal cord explants from chick embryos—technique, 268
  watch glass techniques
    for bone, **235** f.
    for endocrine glands (infantile)
    —human, **241**
    —in moist chamber, **237** ff.
Tissues: preparation for flame photometry, 88
  dry ashing, 93
  nitric acid extraction, 94
Transducer, electrical: for intralumen pressure registration, 119 ff.
  miniature, 121 f.
Tritium oxides: for total body water estimations, 31
Trypanosoma: intracellular cultivation, 281 ff.
Tweens: for esterase and lipase demonstration, 20

U

Urine
  for flame photometry, 88, 93
  NAAP in—estimation, 36 f.
    reagents, 36
    standards, 37
  thiocyanate determination in, 52
Urates: histochemical demonstration, 7
Uric acid
  histochemical demonstration, 7
  removal from tissues for Ca demonstration, 4

V

Vagotomy: insulin-acidity test of, 166
Vitamin A: histochemical demonstration, 13

W

Water, extracellular, *see* Fluid, extracellular
Water, total body: measurement, **31** ff.
  antipyrine for, 31 ff.
    errors, 32
    evaluation, 31
    technique, 32
  NAAP for, 35 ff.
    urinary excretion procedure, 34, 35 f.

Z

Zinc
  histochemical staining for, 5
  -leuco technique (Lison) for peroxidase localization, 15

NAME INDEX

[Page numbers printed in bold face indicate original contributions to this volume.]

A

Adkison, J. L., 197
Agren, G., 166
Alfin-Slater, R. B., 232
Allison, J. B., 47
Allsopp, C. B., 277
Altschule, M. D., 53
Anderson, M. X., 48, 49, 53
Anigstein, L., 279
Armour, J., 146, 148
Armour, J. C., 149
Armstrong, S. H., Jr., 53
Åstrup, T., 208
Axelrod, J., 38

B

Babkin, B. P., 148, 150, 152, 156, 164, 172
Bachrach, W. H., 172
Baker, J. R., 7, 27
Bale, W. F., 67
Ballem, C. M., 172
Bang, F. B., 291
Barnes, B. O., 79
Barnes, R. B., 81, 83, 84, 105
Barnett, H. L., 40, 47
Barrett, R., 278
Bassov, L. V., 123, 148
Bauer, H., 27
Baxter, S. G., 164
Bazett, H. C., 150, 164
Beaumont, W., 124, 148
Behnke, A. R., Jr., 38
Benjamin, F. B., 184
Bennett, A. H., 284, 286, 287
Bennett, A. L., 197
Bensley, R. R., 197
Berenblum, I., 225, 226, 229, 231
Berger, E. Y., 38
Berger, R. E., 279
Bergmann, M., 202, 204, 208
Berliner, R. W., 83, 102, 105
Bernard, Claude, 154, 164
Bernstein, N. O., 154, 164
Berry, J. W., 81, 83, 105
Best, F., 10, 27
Bickel, A., 145, 148
Biesele, John J., 264, **272**, 277
Bills, C. E., 104, 105
Blandlot, N., 124, 148
Blatt, H., 279
Blickenstaff, D., 184, 185
Bliss, C. I., 180, 185
Blocker, T. G., Jr., 277
Bloom, W. A., 258
Boldyreff, W. N., 134, 148, 155, 156, 164
Bollman, J. L., 143, 148, 165
Borghese, E., 233
Bottin, J., 163, 164
Boyd, G. A., 254
Boyd, L. J., 172
Boyden, A. A., 47
Brachet, J., 27
Bradley, 129
Bradley, W. B., 185
Brestkin, M. P., 130, 148
Brimhall, S. D., 152, 165
Brines, J. K., 45, 47
Brodie, Bernard B., **31**, 33
Brody, Daniel A., **109**, 123
Brues, Austin M., 67, **247**, 254, 255, 258, 259, 277
Brunschwig, A., 67, 137, 148
Buchanan, D. L., 254
Bucher, G. R., 197
Buchsbaum, Ralph, 254, **255**, 258
Burch, G. E., 62, 63, 67, 68
Burn, J. H., 165

C

Cain, A. J., 27
Cameron, G., 267, 277, 281, 283
Campbell, D. A., 173
Campbell, W. N., 47
Canti, R. G., 237
Carpenter, E., 234
Carr, C., 68
Cassels, D. E., 48
Chagas, C., 283
Chaikoff, I. L., 68
Chain, E., 225, 226, 229, 231
Chappell, D. G., 83, 105
Chavre, V. J., 196
Chigins, 125
Chinard, Francis P., **38**, 47
Clark, A. J., 69, 78, 79
Claude, A., 274, 277, 280, 291
Code, C. F., 122, 123, **174**, **175**, **176**, **177**, 179, 180, **181**, 185
Coffey, R. J., 158, 165
Cohn, E. T., 67
Cohn, W. E., 67, 254, 255, 277
Colosi, N. A., 165

Compere, E. L., 79
Cope, O., 126, 135, 148
Cordier, R., 27
Cornman, I., 278, 279
Courtice, F. C., 47
Covell, W. P., 165
Cowie, D. B., 67
Craig, J. S., 209, 210
Crandall, L. W., Jr., 48, 49, 53
Crane, E. E., 188, 196
Crider, J. O., 150, 160, 164, 166
Crispell, K. R., 46, 47
Cronvich, J. A., 67
Curtiss, L. F., 255
Cuthbertson, E. M., 67

D

Dackermann, M. E., 231
Danchakoff, V., 268, 277
Danes, B., 280, 288
Danielson, M., 226, 231
Danowski, T. S., 38
Darke, S. J., 208
Davenport, Horace W., **185**, 191, 196
Davidson, J. N., 225, 226, 227, 231
Davies, R. E., 189, 190, 196
Dawson, A. B., 39, 47
de Graaf, Regner, 154, 165
Delrue, G., 187, 196
Dische, Z., 227, 231
Doljanski, L., 258, 259
Donnet, V., 173
Dorris, F., 234
Drager, G. A., 279
Dragstedt, C. A., 126, 148
Dragstedt, L. R., 130, 148, **157**, 159, 165, 173
Draper, G., 277
Drinker, N., 79
Dubbs, Clyde A., 105
Duggar, B. M., 256, 258
Dunning, M. L., 38
Dupertuis, C. W., 277
Duryee, W. R., 259
Dustin, Pierre, 264
Duthie, E. S., 165

E

Earle, Wilton R., **217**, **218**, 219, 221, 277
Eder, Howard A., **47**, **48**
Edwards, C. T., 189, 196
Edwards, L. E., **186**, **187**, 189, 190, **192**, **194**, **196**
Ehrensvärd, G., 205, 206, 208
Eisenman, A. J., 53, 68
Eisenman, A. M., 103, 105
Elftman, A. G., 6, 27
Elftman, H., 6, 27
Elkinton, J. R., 49, 53, 68
Ellis, E. J., 123
Ellis, J. C., 148, 165
Elman, Robert, 30, 48, **152**, 154, 159, 165
Erdmann, R., 244, 245
Evans, H. M., 39, 47
Evans, R. D., 255
Evans, Virginia J., 219, **221**, **225**, 277
Ezell, D., 265, 278, 279

F

Farrell, J. I., 143, 148, 164, 165
Farris, E. J., 288
Feen, B. G., 38
Fell, 214
Fell, Honor B., **233**, **234**, 237, 277, 278
Fellers, F. K., 40, 47
Fellers, F. X., 53, 67
Fenn, W. O., 67
Feulgen, R., 27
Fieser, L. F., 48
Firor, W. M., 288
Fischer, A., 202, 206, 208, 278, 280, 283
Fisher, R. B., 196
Flexner, Louis B., **29**, 38, 67, 68
Fofoid, C. A., 283
Foster, J. P., 152, 165
Frank, O., 109, 115, 123
Frankenberger, Z., 27
Freis, E. D., 49, 53
Frémont, 124, 148
Friedman, M. H. F., **123**, 148, 165, 185
Friedmann, E., 278
Frouin, A., 165
Fullam, E. F., 274, 280, 291

G

Gaillard, Peter J., 214, 215, 234, 240, **241**, 245, 246, 267, 278
Gamble, J. L., 149, 165
Gassul, R., 244, 246
Gaudino, M., 50, 53
Gauer, O., 122, 123
Gautheret, R. J., 206, 208
Gellhorn, A., 38, 56, 67, 68
Gerard, P., 27
Gershbein, L. L., 165, 185
Gey, G. O., 209, 210, 244, 246, 278, 288
Gey, M. K., 209, 210
Gibson, J. G., 29, 39, 47
Giese, A. C., 259
Gilligan, R. D., 53
Giroud, A., 27
Glass, G. B. J., 172
Glasstone, S., 234

Glick, D., 196
Goebel, W., 69, 79
Goldberg, S. L., 126, 143, 148
Goldhaber, G., 258, 259
Goldhaber, P., 278
Goldstein, B., 164, 165
Goldzieher, J. W., 105
Gomori, George, **1**, 27
Gordon, A. H., 205, 208
Gray, J. S., 180, 185, 197
Greco, J., 28
Green, A. A., 79
Green, M. H., 28
Greenberg, D. M., 67
Greenberg, J., 152, 165
Greengard, H., 165
Gregersen, M. I., 39, 43, 47, 48, 52
Grindlay, J. H., 197
Grossman, M. I., 146, 172, **173**, 180, 184, 185
Gruber, C. M., 165
Gruber, C. M., Jr., 165
Guimaraes, F. N., 283
Günther, F., 69, 79

H

Haege, L. F., 68
Hahn, L., 67
Halberstaedter, L., 259
Hald, Pauline M., **79**, 103, 105
Hamilton, J. G., 255
Hanks, J. B., 272, 278
Hanks, J. H., 278
Hansen, A. T., 116, 123
Hanson, M. E., 185
Hardy, M. H., 234
Harington, C. R., 48
Harman, H. H., 197
Harris, R. J. C., 231
Harrison, J. S., 172
Harrison, R. G., 198
Hartman, A. F., 159, 165
Hartwell, J. L., 48
Hastings, A. B., 68, 72, 77, 78, 79, 105
Hawking, F., 283
Heatley, N. G., 225, 226, 231
Heidenhain, R., 124, 141, 148, 154, 156, 165
Heller, V. G., 63, 67
Hepp, O., 30
Hess, O., 165
Hetherington, Duncan C., **209**, 210
Hevesy, G., 38, 67, 255
Hildegard, W., 255
Hinrichs, M. A., 79
Hirsch, G. C., 165
Hodge, H. C., 67
Hoerner, M. T., 163, 165
Hoerr, N. L., 197
Hofer, E., 38
Hogeboom, G. H., 228, 232
Hollander, Franklin, 131, 133, 148, **166**, 168, 171, 172, 173, 177, 185, 197
Holt, W. M., 255
Holton, P., 165
Hood, 81
Hotchkiss, R. D., 27
Houssay, B. A., 164, 165, 187, 197
Howat, H. T., 185
Huebner, W., 69, 79
Hughes, A. F. W., 278, 287, 288
Hughes, Arthur, 264
Hull, W., 229, 231, 252, 255

I

Inlow, W. DeP., 156, 157, 165
Ivers, J. B., 278
Ivy, A. C., 143, 148, 164, 165, 172, 185

J

Jackson, D. E., 151, 165
Jackson, E. B., 255
Jacobson, W., 280
Jacoby, F., 208, 267, 278
Jacquez, J. A., 278
Jemerin, E. E., 131, 133, 148, 167, 171, 172, 173
Jensen, V., 196
Johnson, B. B., 279
Johnson, H. O., 105
Johnson, J. R., 279
Jones, B., 196
Joseph, N. R., 68, 79
Junge, J., 28

K

Kaltreider, N. L., 49, 51, 53, 55, 56, 67
Kamen, M. D., 67
Karzon, D. T., 291
Katsch, G., 145, 148
Kaulberz, J., 185
Kay, G. A., 38
Keisker, H. W., 122, 123
Kelly, F. G., 29, 30
Kelly, F. J., 48
Kerr, S. E., 231
Kingsland, L. C., 255
Kirk, P. L., 226, 229, 231, 252, 255
Kisieleski, W., 258, 259
Klein, E., 144, 148
Klemensieviciz, 124, 148
Klinke, K., 69, 79
Komarov, S. A., 126, 148
Kooreman, P. J., 243, 246
Kóssa, J. v., 3, 27
Kowalski, H. J., 50, 53
Kudryavzeff, N. N., 173

Kühne, W., 165
Kunkel, P., 47

L

Lacquet, A. B., 196
Laidlaw, G. F., 28
Lamson, P. D., 165
Langman, J., 244, 246
Larack, A. M., 232
Laser, H., 278
Lasfargues, E., 205, 209
Laurence, G. D., 232
Lauson, H. D., 47
Lavietes, P. H., 53, 105
Lawrence, J. H., 255
Layton, L., 105
Lea, A. S., 165
Lea, D. E., 256, 259
Leblond, C. P., 27
Leslie, I., 227, 231
Levitt, M. F., 53
Lewert, R. M., 283
Lewis, S. R., 277, 278
Lewis, W. H., 263, 264, 278
Likely, G. D., 218
Lillie, R. D., 28
Lim, R. K. S., 146, 185
Linderstrom-Lang, K., 194, 197
Lison, L., 27, 28
Longmuir, N. M., 196
Lowry, O. H., 105
Ludford, R. J., 263, 279
Luetscher, J. A., Jr., 48
Lundegardh, H., 81, 105
Lykken, L., 105

M

Malmejac, J., 173
Manery, J. F., **53**, 67, 68
Mann, F. C., 126, 143, 148, 152, 165, 197
Mann, F. D., 186, 197
Marinelli, L. D., 61, 68
Markowitz, J., 163, 165
Marks, H. K., 165
Marks, H. P., 185
Marrian, D. H., 278
Martin, A., 291
Martinovitch, Petar N., 234, **237**, **240**
Mayer, P., 11, 28
McCarthy, J. E., 185
McCaughan, J. M., 154, 155, 158, 159, 165
McClelland, J. A. C., 105
McCrea, E. D., 148
McIver, M. A., 149, 165
McLean, Franklin C., **68**, 77, 78, 79
McManus, J. F. A., 28
McMaster, P. D., 154, 166
McNeil, E., 283
McSwiney, B. A., 172
Medawar, P. B., 279
Meister, F. C., 49, 53
Mejbaum, W., 228, 231
Mellanby, E., 234, 237
Mellanby, J., 165
Menten, M. L., 28
Merrell, M., 38, 56, 68
Meschan, I., 120, 123
Messinger, W. J., 38
Meyer, Hertha, **281**, 283
Meyer, K. A., 173
Michel, K., 288
Millen, H. M., 185
Molinelli, E. A., 164, 165
Monges, H., 173
Montgomery, M. L., 68, 165
Moore, F. D., 38
Morgan, J. F., 202, 206, 208, 267, 279
Morris, C. J. O. R., 48
Morse, M., 45, 48
Morton, H. J., 202, 206, 208
Mozer, P., 146
Mullenix, R. B., 126, 148
Murphy, F. T., 163
Murphy, J. S., 291
Murray, Margaret R., **211**, **216**, 279

N

Nachlas, M. M., 28
Nachman, H. M., 48
Nantz, F. A., 279
Naranjo, A., 254
Nasset, E. S., 140, 149
Necheles, H., 134, 148
Neidhardt, H. W., 278
Neuwelt, F., 134, 135, 148
Nickerson, J. L., 43, 48
Niemann, C., 202, 204, 208
Nieset, R. T., 46, 47
Noble, R. L., 172, 173
Noble, R. P., 39, 48
Nowinski, W. W., 231, 280

O

Oberling, C., 291
Oehlenschlager, V., 208
Ogur, M., 228, 231
Okamoto, K., 28
Olitzki, P. K., 283
Olivo, O. M., 279
Olson, W. H., 134, 148
Ormsbee, R. A., 272, 278, 279
Orr, M. F., 280
Orr, M. K., 48
Osserman, E. F., 38
Ottolenghi, L., 226, 229, 232
Owings, J. C., 244, 246

P

Pace, Nello, **37**, 38
Paff, G. H., 263, 279
Painter, E. E., 38
Painter, J. T., 265, 279
Parker, R. C., 202, 206, 208, 262, 267, 270, 271, 279, 281, 283
Parks, T. D., 105
Patterson, E. K., 231
Patterson, W. B., 197
Paul, H., 63, 67
Pavlov, I. P., 124, 125, 131, 142, 148, 150, 156, 165
Peacock, W. C., 29, 30, 48
Penman, R., 47
Percival, G. H., 69, 79
Perry, W. L. M., 287, 288
Petermann, M. L., 232
Peters, J. P., 48
Peterson, E. R., 216, 279
Petralia, S., 279
Phillips, R. A., 48
Pickels, E. G., 277, 291
Pieper, G. F., 257, 259
Pierce, C., 277
Pochin, E. L., 48
Pogogeff, I. A., 216, 279
Pomerat, C. M., **198**, 208, **260**, **261**, **262**, 265, **266**, 271, 274, **275**, 277, 278, 279, 280
Popper, H., 13, 28
Porosowska, Y., 38
Porter, B., 46, 47
Porter, Keith R., 263, 274, 277, 278, 280, **288**, 291
Pratt, J. H., 163, 165

Q

Quigley, J. P., **106**, **109**, 120, 123, 143, 146, 161, 166, 173

R

Ramsey, A. J., 164, 166
Ramsey, H. J., 277
Rathbun, C., 38
Rathbun, E. N., 254, 277
Rawson, R. A., 39, 48
Rawson, R. W., 255
Reaser, P. B., 67, 68
Reeve, E. B., 44, 45, 48
Rehm, W. S., 30, 186, 197
Reid, A. F., 48
Revell, D. G., 152, 166
Ricamo, R., 279
Richards, Oscar W., **284**, 288
Richardson, 81
Rienhoff, W., 234
Riggs, B. C., 193, 197
Rioch, D., 268, 280
Robertson, C. R., 185
Robison, R., 234, 237
Romana, C., 283
Rose, G. G., 280
Rose, J. M., 280
Rosen, G., 228, 231
Rosenbaum, J. D., 53
Rosiere, C. E., 184
Ross, H. E., 228, 232
Rous, P., 154, 166
Russell, F. C., 255
Russell, S. J. M., 45, 48

S

Sabin, A. B., 283
Sanders, M., 216
Sandweiss, D. J., 185
Sanford, Katherine K., 218, **219**, 280
Savitch, W. W., 130, 148
Scatchard, G., 50, 53
Schachter, M., 173
Scheinberg, I. H., 50, 53
Schilling, E. L., 219
Schlutz, F. W., 48
Schmidt, C. R., 166
Schmidt, G., 225, 227, 229, 232
Schmitz, R. L., 67
Schneider, W. C., 225, 228, 230, 232
Schofield, B., 185
Schreiber, J., 110, 123
Schultz, A., 13, 28
Schultze, W. H., 28
Schwartz, I. L., 53
Scott, V. B., 153, 156, 157, 159, 161, 166, 197
Sehrt, E., 28
Seligman, A. M., 28
Semionova, G. T., 149
Senn, N., 158, 166
Seraidarian, K., 231
Sfortunato, T., 226, 229, 232
Shannon, J. A., 38
Sharick, P. R., 173
Shaw, D. T., 255
Sheline, G. E., 68
Shelton, E., 280
Signorotti, B., 252, 255
Simanovskaia, S., 124, 148
Simms, H. S., 216
Simon-Reuss, I., 278
Simonsen, D. H., 30, 48
Siri, W. E., 68
Sise, H. S., 38
Slabeycius, M., 280
Soberman, R., 31, 32, 38
Sokalchuk, A., 47
Squire, J. R., 48
Stadie, W. C., 193, 197
Starling, E. H., 164
Steele, J. M., 38

Steele, R., 226, 229, 232
Steigmann, F., 173
Stein, I. F., Jr., 173
Stein, J., 172
Stettin, D., 197
Stewart, C. P., 69, 79
Stewart, J. D., 48
Still, E. U., 187, 197
Stjernholm, R., 208
Stone, G. C. H., 105
Stone, H. B., 244, 246
Storer, E. H., 173
Stout, A. P., 216, 279
Strangeways, D. H., 234
Strangeways, T. S. P., 234, 235
Straub, W., 69, 79
Stroud, Agnes N., 247, 254
Svihla, G., 255, 258, 259
Swann, M. M., 287, 288

T

Taffel, M., 49, 53
Tchakhotine, S., 258, 259
Templeton, R. D., 146, 173
Thannhauser, S. J., 225, 227, 229, 232
Thomas, J. E., 125, 138, **149**, 150, 160, 161, 164, 165, 166
Thompson, H. P., 263, 280, 291
Thompson, O. E., 155, 164
Thomson, D., 233, 234
Thorn, G. W., 47
Thornton, T. F., Jr., 173
Threefoot, S. A., 67, 68
Tomlin, S. G., 291
Tonkin, I. M., 283
Tracy, M. M., 254
Trendelenburg, P., 69, 79
Trowell, O. A., 267, 278
Tuckerman, L. B., 160, 166

U

Ulloa-Gregori, O., 280
Utamura, M., 28

V

Vanamee, P., 291
van Dyke, H. B., 72, 79
Varney, P. L., 85, 105
Veall, N., 48
Visscher, M. B., 68
Voit, K., 27
Volborth, G. W., 173

W

Wachstein, M., 28
Wagner, R. H., 30
Wallace, W. M., 105
Walther, A. A., 166
Wang, C. C., 165
Wang, J. C., 68
Warren, S., 255
Waterhouse, A., 38
Watman, R. N., 140, 149
Waymouth, Charity, **201, 225, 226.** 227, 231, 232, 263
Webster, D. R., 149, 172
Weichselbaum, T. E., 85, 105
Weinstein, V. A., 173
Welham, W. C., 38
Wesson, L. G., 255
Wetterer, E., 122, 123
Whalley, H. K., 105
Whipple, G. H., 39, 47
White, Philip R., **201, 202, 205, 206.** 207, 208, 209, 280
Wiggers, C. J., 109, 123
Wilander, O., 166
Wilde, W. S., 67
Williams, R. H., 38
Williams, V. Z., 105
Willmer, E. N., 225, 232, 267, 278 280
Wilson, D. L., 68
Windaus, A., 28
Winkelstein, A., 173
Winkler, A. W., 51, 53, 68
Wood, A. B., 123
Wood, E. H., 123
Wood, F. D., 283
Woodward, E. R., 146

X

Xavier de Oliveira, M., 283

Z

Zak, F. G., 28
Zelle, K., 197
Zinsser, H. H., 79
Zirkle, R. E., 258, 259
Zweig, M., 173

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, * MYSORE. *
LIBRARY

Checked by 23/11/80.

7-8-80

21 8 89

8-5-92

CHECKED
2008

VERIFIED
2013

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, * MYSORE. *
LIBRARY

CFTRI-MYSORE
3538
Methods in medic.